

Studies on α -Adrenoceptors in the Female Rabbit Urethra

by

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From the Department of Clinical Pharmacology,
University of Lund, Sweden

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Title and subtitle STUDIES ON α -ADRENOCEPTORS IN THE FEMALE RABBIT URETHRA			
Abstract The α-adrenoceptors (α-ACs) in the female rabbit bladder base and urethra were studied by means of radioligand binding technique. In addition, functional experiments on isolated female rabbit urethra were performed as a complement to the binding studies. The presence of α-ACs in a crude membrane preparation obtained from the bladder base and urethra was demonstrated, using the non-selective α-AC antagonist ^3H-dihydroergocryptine (^3H-DHE) as a marker for α-ACs. Through both saturation studies, using ^3H-prazosin and ^3H-rauwolscine, and inhibition experiments of ^3H-DHE binding by prazosin and rauwolscine, the proportions of α_1- and α_2-ACs in the membrane preparation were estimated to approximately 25 and 75%, respectively. In functional studies it was found that both α_1- and α_2-ACs are located postjunctionally in the rabbit urethra. The sensitivity of the urethra to exogenously administered noradrenaline (NA) was increased by in vivo estrogen treatment. The results from radioligand binding studies suggest that this was due to an increase in the number of α_2-ACs. This effect of estrogen might contribute to the beneficial effects on stress urinary incontinence seen in women after estrogen treatment. The effects of nifedipine, verapamil and diltiazem were investigated on NA-induced contractions and electrically evoked fractional efflux of ^3H-NA. Some of the differences in effects between verapamil, and nifedipine and diltiazem on these processes might be explained by the effects on α-ACs found for verapamil in radioligand binding studies. The antimuscarinic agent atropine was found to potentially inhibit NA-induced contractions and α-AC radioligand binding. This was not found for scopolamine. The effects of verapamil and atropine on α-ACs underline the importance of using drugs in appropriate concentrations in order to obtain selective effects of the drugs.			
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To Liselotte

The present thesis is based on the following papers, which in the text will be referred to by their Roman numerals (I-V).

- I B. Larsson: Demonstration of α -adrenoceptors in the rabbit bladder base and urethra with ^3H -dihydroergocryptine ligand binding. Acta pharmacol et toxicol 52:188-194, 1983
- II K.-E. Andersson, B. Larsson, C. Sjögren: Characterization of the α -adrenoceptors in the female rabbit urethra. Br J Pharmacol (In press)
- III B. Larsson, K.-E. Andersson, S. Batra, A. Mattiasson, C. Sjögren: Effects of estradiol on norepinephrine-induced contraction, alpha-adrenoceptor number and norepinephrine content and release in the female rabbit urethra. J Pharmacol Exp Ther (Submitted for publication)
- IV B. Larsson, E. D. Högestätt, A. Mattiasson, K.-E. Andersson: Differential effects of nifedipine, verapamil, and diltiazem on noradrenaline-induced contractions, adrenergic transmitter release, and alpha-adrenoceptor binding in the female rabbit urethra. Naunyn-Schmiedeberg's Arch Pharmacol (Accepted for publication)

- V. B. Larsson, K.-E. Andersson, A Mattiasson:
Influence of antimuscarinics on alpha-adrenoceptors
in the female rabbit urethra. Acta Physiol Scand
(Accepted for publication)

CONTENTS

INTRODUCTION.....	1
Receptors.....	1
Adrenoceptors.....	2
Alpha-Adrenoceptors.....	2
Subclassification.....	2
Localization.....	4
Controversies.....	5
Radioligand binding studies.....	6
Bladder and urethral smooth muscle.....	9
OBJECTIVES OF THE PRESENT INVESTIGATION.....	11
MATERIALS AND METHODS.....	13
Animals.....	13
Radioligand binding studies.....	13
Functional studies.....	15
Recording of mechanical activity.....	15
Release studies.....	15
Measurement of noradrenaline content.....	16
Calculations.....	16
RESULTS.....	17
Study I.....	17
Study II.....	19
Study III.....	22
Study IV.....	26
Study V.....	28
GENERAL DISCUSSION.....	30
ACKNOWLEDGEMENTS.....	39
REFERENCES.....	41
ENCLOSURES I-V	

INTRODUCTION

Receptors

The concept of a receptive mechanism for drugs in the effector organ was probably first expressed by Langley about a century ago (Langley 1878, 1905, 1906). He suggested (Langley 1905) that "in all cells two constituents at least are to be distinguished, a chief substance, which is concerned with the chief function of the cell as contraction and secretion, and receptive substances which are acted upon by chemical bodies and in certain cases by nervous stimuli. The receptive substance affects or is capable of affecting the metabolism of the chief substance". Based on the work of Langley, and on his own studies of chemotherapeutic agents, Ehrlich (1913) later coined the term "receptors".

Ehrlich was aware that a small structural modification of a substance could have large effects on the therapeutic action of the substance. He writes (Ehrlich 1913): "all the derivatives of arsenic which contain arsenic in the pentavalent form - i.e. in the fully saturated form - do not bring about any therapeutic action, but that this only commences when the arsenic group exists in the unsaturated condition corresponding to the trivalent radical". This insight presaged the concept of drug affinity to receptors. Although many experimental designs allow only the potency of a drug to be determined, the order of potency of a series of drugs for a given receptor has proven itself very useful

in receptor research. (For reviews of the historical perspectives of the evolution of the receptor concept and receptor theories, see Waud 1968, DeMeyts and Rousseau 1980, Ruffolo 1982).

Adrenoceptors

Dale (1906) used the idea of a "receptive substance for adrenaline" when discussing his finding that ergot largely abolished the excitatory effects of adrenaline, while leaving the inhibitory responses unaffected. Until 1948 the adrenoceptors were considered to be either excitatory or inhibitory. However, this was questioned by Ahlquist (1948). Studying the order of potency of a series of 6 sympathomimetic amines, Ahlquist found one order of potency for the 6 sympathomimetic amines on vasoconstriction, excitation of the uterus and ureters, contraction of the nictitating membrane, dilatation of the pupil and inhibition of the gut, whereas a different potency order was found on such functions as vasodilation, inhibition of the uterus and myocardial stimulation. Based on these observations Ahlquist suggested that the adrenoceptors should be classified into alpha- and beta-adrenoceptors.

Alpha-adrenoceptors

Subclassification

After the subclassification of the beta-adrenoceptor into 2 subgroups (Lands et al. 1967), it was not surprising that the alpha-adrenoceptor was suggested also to be subdivided into two groups, the α_1 - and α_2 -adrenoceptors. The subdivision of these receptor

subtypes have been made according to anatomical location (Langer 1974), functional characteristics (Berthelsen and Pettinger 1977) and more recently exclusively on pharmacological grounds (for reviews, see Wikberg 1979, Starke 1981a, Langer and Shepperson 1981; Table 1). The development of new agents selective for the respective adrenoceptor subtype and the introduction of radio-ligand binding studies have added many new aspects of the alpha-adrenoceptor; greatly stimulating research in this field.

<u>Agonist</u>		<u>Antagonist</u>
Phenylephrine	α_1	Prazosin
Noradrenaline	$\alpha_1 = \alpha_2$	{ Phentolamine Dihydroergocryptine
Clonidine	α_2	Rauwolscine

Table 1. Suggested selectivity of some agonists and antagonists for the alpha-adrenoceptor subtypes.

Localization

Alpha-adrenoceptors have been found in various tissues such as fat cells, blood cells, heart, smooth muscle, and nervous tissue. The function of these receptors may vary depending on their location. For example alpha-adrenoceptors located on vascular smooth muscle are involved in vasoconstriction (Moran 1975), whereas on terminal noradrenergic axons they mediate inhibition of noradrenaline release (Langer 1974, Starke 1981b).

A structure in which the alpha-adrenoceptor has been greatly studied is the neuro-muscular junction. There, the prejunctional receptor, which modulates transmitter release, is suggested to be of the α_2 -type (Langer 1974, Starke and Docherty 1980), whereas the picture of the postjunctional alpha-adrenoceptor has been more complicated (Starke 1981a, Timmermans and van Zwieten 1981, Timmermans and van Zwieten 1982). Originally it was suggested that the postjunctional alpha-adrenoceptor was of the α_1 -type (Langer 1974). However, the possibility that also another type of alpha-adrenoceptor could be located postjunctionally was suggested by Moulds and Jauernig (1977), Jauernig et al. (1978) and Drew and Whiting (1979). Later a great many studies have appeared suggesting the existence of a mixture of postjunctional α_1 - and α_2 -adrenoceptors in the same tissue preparation (Docherty et al. 1979, Timmermans et al. 1979, U'Prichard and Snyder 1979, Timmermans and van Zwieten 1980, Constantine et al. 1980, Langer et al. 1980, DeMey and Vanhoutte 1981).

Recently it was proposed that postjunctional alpha-adrenoceptors are predominantly of the alpha₂-type in the isolated feline middle cerebral artery (Skärby et al. 1983).

When studying the autoperfused hindlimb of the dog, Langer et al. (1980) observed that the response to electrical stimulation could be blocked by prazosin, whereas this drug was less effective in inhibiting the response to exogenously administered noradrenaline (NA). This led them to suggest that the postjunctional alpha₁-adrenoceptor was to be found within the neuro-muscular junction, whereas the alpha₂-adrenoceptor was located primarily extrajunctionally in this preparation.

Controversies

Although the pharmacological classification of the alpha-adrenoceptors into the alpha₁- and alpha₂-subgroups is widely used today, some authors report that their experimental data do not fit into this classification system. They propose alternative explanations such as further division into more subgroups (Wood et al. 1979, Ruffolo et al. 1980, Ruffolo et al. 1981, Cheung et al. 1982, Ruffolo et al. 1982) or the occurrence of an alpha-adrenoceptor unable to differentiate between substances that in other tissues act preferentially on alpha₁- or alpha₂-adrenoceptors (Skärby et al. 1983), and the introduction of a "gamma" receptor within the neuro-muscular junction (Hirst and Neild 1980, Neild and Zelcer 1982). Controversies in the classification of vascular alpha-

adrenoceptors have recently been reviewed (McGrath 1982).

Radioligand binding studies

Most of the information about the alpha-adrenoceptor has been obtained through studies applying conventional pharmacological techniques. However, by these techniques the interaction between the drug (ligand) and the receptor is not studied directly, but involves in addition amplifier and effector systems before the final response is measured (Fig 1). Although a physiological response can be measured upon receptor stimulation, it may be difficult to deduce what is actually taking place at the receptor site. Some of these difficulties have been surmountable by the introduction of the radioligand binding technique. However, the possibility to label "receptors" that do not have a physiological function (Cuatrecasas and Hollenberg 1975, Snyder et al. 1975) makes it important to use radioligand binding studies in connection with conventional pharmacological techniques.

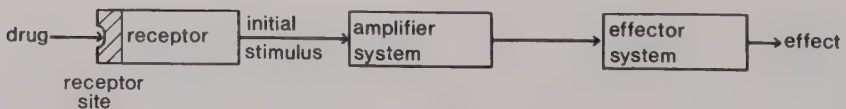


Fig 1. Schematic drawing of drug-receptor binding and subsequent cellular events leading to a biological effect.

The first successful attempts to label the alpha-adrenoceptor with a radioactively labelled ligand were reported in 1976 by Williams and Lefkowitz (1976) and Greenberg et al. (1976). Since then a great many reports have been published which support the division of the alpha-adrenoceptors into the α_1 - and α_2 -subgroups. In these studies a variety of principally different radioligands such as selective and non-selective antagonists as well as selective agonists have been used (Greengrass and Bremner 1979, Tanaka and Starke 1979, Hoffman and Lefkowitz 1980, Lavin et al. 1981, Rouot et al. 1982). α_2 -adrenoceptor agonists have been found to bind to a high and a low affinity binding site of the α_2 -adrenoceptor. The presence of high concentrations of guanine nucleotides appears to transform the high affinity receptor sites into the low affinity form (Hoffman et al. 1980). Presumably ^3H -labelled α_2 -adrenoceptor agonists will label only the high affinity portion of the α_2 -adrenoceptors, since, most frequently, only low concentrations of ^3H -labelled agonists are used in radioligand binding studies. Furthermore, binding of agonists to α_2 -adrenoceptors was found to be sensitive to the ion composition of the incubation medium (Glossman and Presek 1979). Consequently these factors have made the interpretation of agonist binding data somewhat difficult. However, antagonists have not been found to be as sensitive as agonists to such factors, and are therefore often preferable in radioligand binding studies. Among the radiolabelled alpha-adrenoceptor

antagonists most frequently used in radioligand binding studies are the selective α_1 -adrenoceptor antagonist prazosin, the selective α_2 -adrenoceptor antagonists yohimbine and rauwolscine and the non-selective α -adrenoceptor antagonist dihydroergocryptine.

Apart from a radioligand, one needs a suitable receptor population in which to study the interaction between ligand and receptor. Such receptor preparations have been obtained from many different tissues, such as brain (Hornung et al. 1979), heart (Karliner et al. 1979), liver (Clarke et al. 1978), fat cells (Berlan and Lafontan 1980), platelets (Newman et al. 1978), and smooth muscle (Williams et al. 1976). Moreover, several criteria need to be fulfilled in order to claim that a receptor has been successfully labelled. Some of the most important are:

The binding of ligand to receptor should be saturable, since there can only be a finite number of receptors per unit tissue.

The binding of ligand to receptor should be reversible, if the effect of the ligand is reversible.

For binding to α -adrenoceptors, the expected order of potency for three well known catecholamines should be, adrenaline > noradrenaline >> isoprenaline. Furthermore, stereoselectivity is to be expected since the (-)-isomers are more effective in eliciting a

functional response than their respective (+)-isomers.

For more detailed information about the radioligand binding technique, see Williams and Lefkowitz (1978a), and Yamamura et al. (1978).

Bladder and urethral smooth muscle

Much of our knowledge about the alpha-adrenoceptor originates from studies of smooth muscle, both in vivo and in vitro. This is probably so because the responses to receptor stimulation can readily be measured. The application of radioligand binding technique to smooth muscle preparations has therefore been an important step in the progress of alpha-adrenoceptor research. The bladder and urethra are smooth muscle preparations that has been used for mechanical activity studies for a long time. Elliott (1905), and Dale (1906) found that the bladder base and urethra responded with contraction to stimulation of the hypogastric nerve, or systemic administration of adrenaline, while the bladder dome responded with relaxation to these stimuli. These effects have been attributed to a functional predominance of alpha-adrenoceptors in the bladder base and urethra and of beta-adrenoceptors in the bladder dome (Edvardsen and Setekleiv 1968, Salimi et al. 1969, Khanna et al. 1981, see Andersson and Sjögren 1982). In accordance, Levin and Wein (1979) found a greater number of alpha-adrenoceptors in the bladder base as compared to the bladder dome, while the opposite was true for beta-adrenoceptors. It should be noted that the

shift in response to NA administration shows a distinct change at the level of the ureteral orifices (Levin et al. 1980a). Thus, the bladder base and urethra are thought to work as a functional unit, and upon stimulation of sympathetic nerves an alpha-adrenoceptor mediated contraction is produced. The function of the alpha-adrenoceptors in the bladder base and urethra has clinical implications since sympathetic tone is considered to be of importance for the maintenance of intraurethral pressure (Awad and Downie 1976, Downie and Awad 1976, see Andersson and Sjögren 1982), and hence to urinary continence.

OBJECTIVES OF THE PRESENT INVESTIGATION

The alpha-adrenoceptors in the female rabbit bladder base and urethra were studied in the present investigation. This has been done mainly by radioligand binding technique. Functional experiments on isolated female rabbit urethra have been performed as a complement to the binding studies. The investigation has been made on the assumption that two alpha-adrenoceptor subtypes exist, the α_1 - and the α_2 -adrenoceptors, which can be separated pharmacologically by drugs selective for the respective subtype.

The aims of the present investigations were:

Study I. To demonstrate the presence of alpha-adrenoceptors in a crude membrane preparation from the female rabbit bladder base and urethra with radioligand binding technique.

Study II. To determine, with radioligand binding technique, the proportions of α_1 - and α_2 -adrenoceptors in a crude membrane preparation of the female rabbit bladder base and urethra, and to correlate the result with mechanical activity studies.

Study III. To study possible mechanisms behind the beneficial effects of estrogens in the treatment of stress urinary incontinence.

Study IV. To probe for differential effects of various calcium entry blockers on nerve and smooth muscle tissue, and to examine possible effects of these substances on alpha-adrenoceptors.

Study V. To study the interaction of some antimuscarinics with alpha-adrenoceptors.

MATERIALS AND METHODS

Animals

Female albino rabbits of the Danish Land race, weighing 2.4 - 3.4 kg, were used throughout the studies except in some experiments in study III where female Dutch rabbits (1.5 - 2.4 kg) were used. The animals were sacrificed and the abdominal cavity opened. The bladder and urethra were dissected free from the vaginal wall down to the urethrovaginal junction and removed en bloc.

Radioligand binding studies

The rabbit bladder base and urethra were cut open, and cleaned of mucosa, adherent fat, and connective tissue. The remaining tissue was used to prepare a crude membrane preparation, which was employed in the radioligand binding studies as the source of alpha-adrenoceptors.

As a marker for alpha-adrenoceptors ^3H -dihydro-alpha-ergocryptine (^3H -DHE) was used in all studies. In addition, ^3H -prazosin and ^3H -rauwolscine were used to label α_1 - and α_2 -adrenoceptors, respectively, in study II. Phentolamine was used throughout to define non-specific binding.

As discussed previously, there appears to be a distinct change in the muscular response of the bladder to adrenergic nerve stimulation at the level of the ureteral orifices. The responses to such stimuli is

inhibitory in the bladder dome and excitatory in the bladder base (Elliott 1905, Dale 1906). Hence both the bladder base and urethra contract upon adrenergic nerve stimulation. Since the amount of tissue available from the female rabbit urethras was too small to allow extensive radioligand binding studies, and since the female rabbit bladder base and urethra can be considered a functional unit with respect to alpha-adrenoceptor activity, both the bladder base and urethra were used in the radioligand binding studies (Fig 2). Although this approach considerably increased the amount of tissue available, the radioligand binding studies were still hampered from the difficulty in obtaining sufficient amount of tissue.

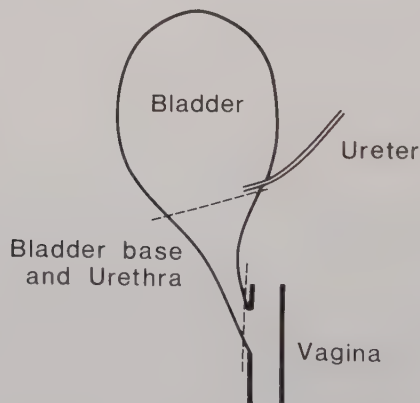


Fig 2. Schematic drawing of the rabbit bladder and urethra. The part of the rabbit bladder base and urethra used in the binding assays is indicated.

Functional studies

Recording of mechanical activity

After the urethras had been trimmed of periurethral fat and connective tissue, either urethral rings (2-4 per urethra, studies II-V) or intact urethras (study III) were transferred to temperature controlled organ baths containing Krebs solution. Subsequently the preparations were suspended for measurement of mechanical activity.

In mechanical activity studies on urethral ring preparations, tachyphylaxis was frequently encountered. The tachyphylaxis was especially pronounced when high concentrations of agonist were used. This prevented us from performing extensive studies that could be analysed according to the method of Arunlakshana and Schild (1959). Instead, either EC_{50} -values from cumulative concentration-response curves were compared, or the inhibitory effect of various substances tested on a single submaximum concentration of agonist.

Release studies

The rabbit urethra was cut open longitudinally, and the urethral mucosa and adherent tissue removed. Two longitudinal pieces of the urethral smooth muscle were dissected from each urethra. The preparations were preloaded with 3H -NA and subsequently placed in temperature controlled organ baths perfused with Krebs solution. The preparations were stimulated electrically and the efflux of 3H determined.

Measurement of NA content

The rabbit bladder base and urethra was cut open longitudinally and the smooth muscle cleaned of mucosa and adherent tissue. Determination of the NA content in the urethra and trigone was performed essentially as described by Eriksson and Persson (1982), using HPLC with an electrochemical detector.

Calculations

Statistical analysis of the results was performed by means of Student's t-test. The 0.05 level of probability was accepted as significant. The results shown in tables and figures are expressed as mean $\pm$ SEM. IC₅₀- and EC₅₀-values were determined graphically from the curves. In radioligand binding studies K_i-values from inhibition studies were determined by the method of Cheng and Prusoff (1973), while the dissociation constant, K_D, for substances inhibiting ³H-DHE binding in saturation experiments were calculated according to Furchgott (1967) (as quoted by Williams and Lefkowitz 1977). Linear least square regression analysis was used where appropriate. Outliers were checked for by Dixon's gap test (Bliss 1967).

RESULTS

I. Demonstration of alpha-adrenoceptors in the rabbit bladder base and urethra with ^3H -dihydroergocryptine ligand binding

The binding of ^3H -DHE was found to be saturable with increasing concentrations of the radioligand. Analysis of the saturation data by a Scatchard plot (Scatchard 1949) yielded a straight line, suggesting the existence of a homogenous population of binding sites. The dissociation constant, K_D , was found to be 1.1 nM, and the total amount of receptors, B_{max} , estimated to 73 fmol/mg protein.

Rate constants for association and dissociation were $3 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$, and $2 \times 10^{-2} \text{ min}^{-1}$, respectively. The kinetically derived dissociation constant for ^3H -DHE was found to be 0.7 nM which is similar to that found in the saturation studies (1.1 nM) and inhibition studies with cold DHE (1.4 nM). The relative affinities of a series of agonists for the ^3H -DHE binding sites were (-)-adrenaline > (-)-noradrenaline > ($\pm$)-isoprenaline (Fig 3). Furthermore, phentolamine was much more potent than propranolol in inhibiting ^3H -DHE binding.

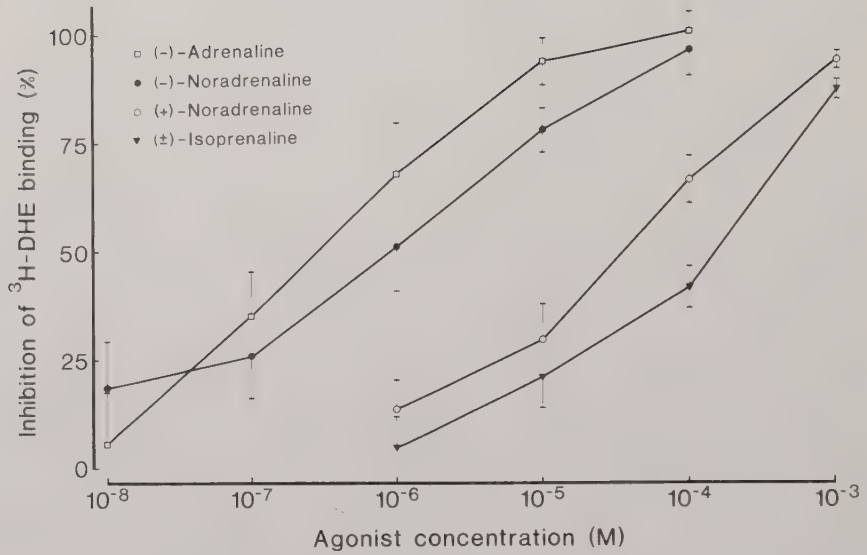


Fig 3. Inhibition of ^3H -DHE binding by various adrenoceptor agonists. Membrane suspensions were incubated with 1 nM ^3H -DHE in the presence of the indicated concentrations of the agonists. The amount of ^3H -DHE binding inhibited by the various concentrations of the drugs were calculated as per cent of specific binding, which was defined as binding exceeding that in blanks containing 10^{-5} M phentolamine. Values represent mean $\pm$ SEM of 3 separate experiments, each performed in duplicate.

II. Characterization of the α -adrenoceptors in the female rabbit urethra

This investigation was designed to evaluate the proportion of α_1 - and α_2 -adrenoceptors in the crude membrane preparation obtained from female rabbit bladder base and urethra, described in study I. In addition, mechanical activity studies were performed on urethral ring preparations.

Radioligand binding studies

Binding studies were performed using ^3H -DHE, ^3H -prazosin and ^3H -rauwolscine. These experiments revealed an α_1 (^3H -prazosin)- and α_2 (^3H -rauwolscine)-adrenoceptor population of 19 ± 4 fmol/mg protein (27 %) and 51 ± 6 fmol/mg protein (73 %), respectively. The sum of receptors determined by ^3H -prazosin and ^3H -rauwolscine (70 fmol/mg protein, 100 %) is in close agreement with the number found for ^3H -DHE (76 fmol/mg protein).

In inhibition studies, biphasic displacement curves were obtained when prazosin and rauwolscine were used to inhibit specific ^3H -DHE (1 nM) binding (Fig 4). In addition, the inhibitory effects of 3×10^{-8} M prazosin and 3×10^{-7} M rauwolscine was found to be largely additive.

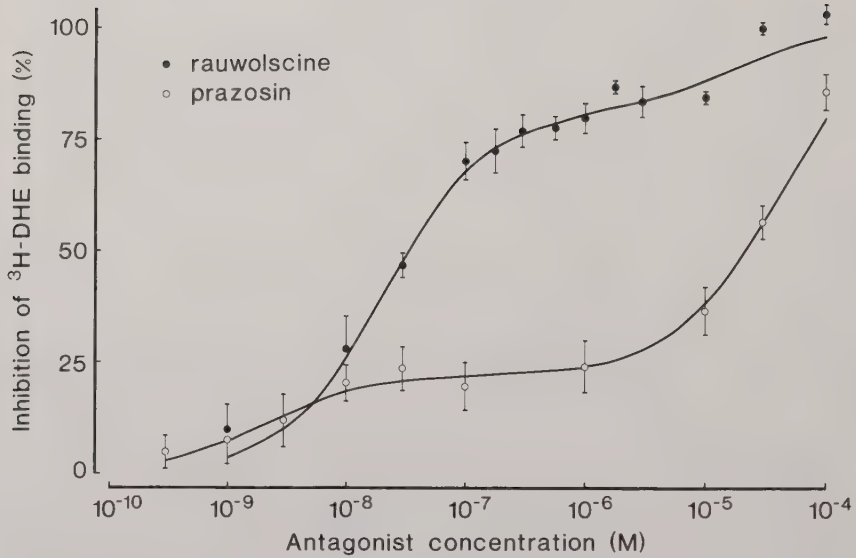


Fig 4. Inhibition of ^3H -DHE binding by prazosin and rauwolscine. Membrane suspensions were incubated with 1 nM ^3H -DHE in the presence of the indicated concentrations of the antagonists. The results are shown as per cent inhibition of specific binding. $n=6$ for both antagonists. Assuming binding to two different binding sites, the lines were drawn according to the equation:

$$\% \text{ inhibition} = \frac{S_1 \times (L)}{K_1 + (L)} + \frac{S_2 \times (L)}{K_2 + (L)}$$

where S_1 and S_2 are the proportions of the respective binding sites, estimated from the plateau of the inhibition curves. K_1 and K_2 are the IC_{50} -values for the inhibition to the respective binding sites. (L) is equal to the antagonist concentration used to inhibit ^3H -DHE binding. For further explanation, see study II.

Mechanical activity studies

The inhibitory effects of prazosin (10^{-6} M) and rauwolscine (1.5×10^{-6} M) were evaluated on contractions induced by the selective α_1 -adrenoceptor agonist phenylephrine (PE) (7.9×10^{-6} M), the selective α_2 -adrenoceptor agonist clonidine (10^{-7} M and 10^{-6} M), and the non-selective α -adrenoceptor agonist noradrenaline (NA) (7.8×10^{-6} M). The results showed that prazosin was more effective in inhibiting PE-induced contractions than those induced by clonidine. The inhibitory effect of prazosin on NA-induced contractions was between those of PE and clonidine. However, when rauwolscine was used to inhibit these agonist-induced contractions, the potency order was reversed (Table 2).

Table 2.

<u>Inhibition of agonist-induced contractions by prazosin (10^{-6} M)</u> <u>and rauwolscine (1.5×10^{-6} M).</u>				
	<u>Prazosin</u>	(n)	<u>Rauwolscine</u>	(n)
	(%)		(%)	
Phenylephrine (7.9×10^{-6} M)	89 ± 4	(6)	34 ± 5	(11)
Noradrenaline (7.8×10^{-6} M)	84 ± 7	(6)	52 ± 4	(7)
Clonidine (10^{-6} M)	69 ± 5	(5)	65 ± 8	(5)
Clonidine (10^{-7} M)	13 ± 4	(6)	100	(6)

III. Effects of estradiol on norepinephrine-induced contraction, alpha-adrenoceptor number and norepinephrine content and release in the female rabbit urethra

The effects of estradiol on NA-induced contraction, alpha-adrenoceptor number and NA content and release were studied in the female rabbit urethra in an attempt to shed light upon the causes to the beneficial effects of estrogens in the treatment of stress urinary incontinence in postmenopausal women (Salmon et al. 1941, Eckerling et al. 1972).

In experiments performed on isolated rabbit urethral ring preparations, the EC_{50} value for NA was significantly decreased ($p < 0.01$) after estrogen treatment (polyestradiol phosphate (Estradurin) 3 mg i.m. 4-5 days before sacrifice). The difference in EC_{50} values for NA between the control and estrogen treated group remained approximately constant in the presence of 10^{-5} M cocaine (Fig 5). In isolated perfused rabbit urethra the EC_{50} value for NA was significantly reduced ($p < 0.001$) for longitudinal tension after estrogen treatment, whereas this was not seen for resistance to flow.

No significant difference in the amount of electrically evoked fractional efflux of 3H -NA was found between the control and estrogen treated groups. In addition, no differences were seen in the ability of clonidine (10^{-6}

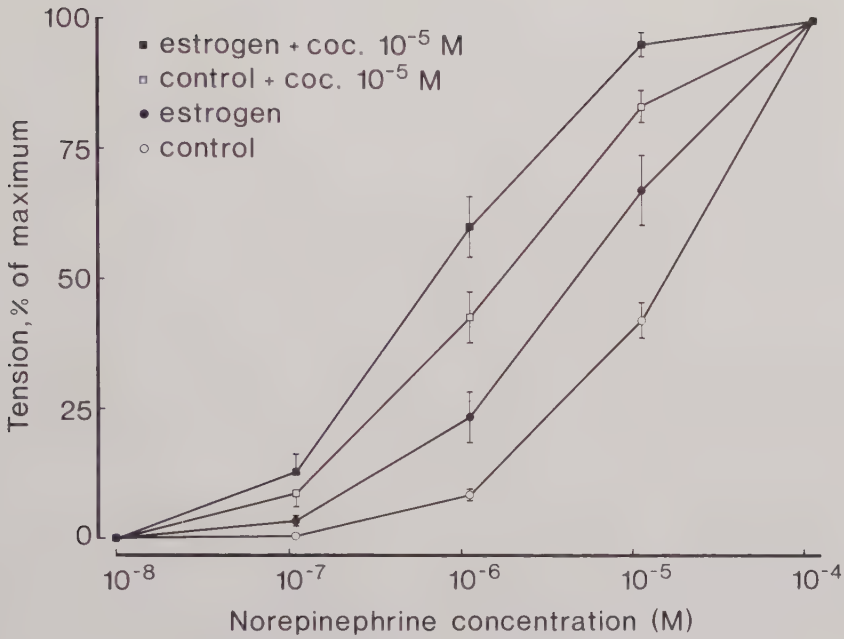


Fig 5. Concentration-response curves for norepinephrine in ring preparations from control and estrogen treated rabbit urethras in the presence or absence of 10^{-5} M cocaine. All responses were recorded in the presence of 3×10^{-7} M propranolol. No further contraction was recorded with 10^{-3} M norepinephrine in any of the groups.

M) and rauwolscine (10^{-7} M) to affect the electrically evoked fractional efflux of ^3H -NA between the two groups investigated.

The total content of NA in the urethra was not changed after estrogen treatment. However, if the NA content in the urethra was calculated per mg wet weight of tissue, the NA content was approximately reduced to half in estrogen treated animals. A similar change in NA content per mg wet weight of tissue was found in the trigone.

The radioligand binding studies revealed a 2.4 fold increase in the number of alpha-adrenoceptors after estrogen treatment ($p < 0.001$), with no change in the affinity of ^3H -DHE for the alpha-adrenoceptors (Fig 6). This was found to be largely attributable to a selective increase in the number of α_2 -adrenoceptors, as revealed from prazosin inhibition curves. In addition, preliminary experiments showed no difference in the ability of NA to inhibit ^3H -DHE binding in estrogen treated animals as compared to controls.

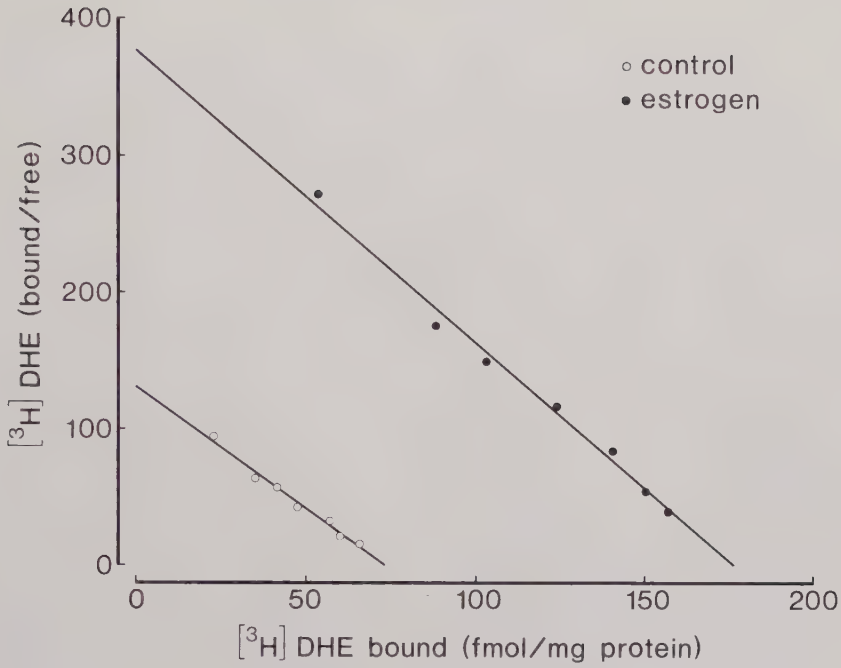


Fig 6. The specific binding of ^3H -DHE to rabbit bladder base and urethral membranes for control and estrogen treated rabbits are given as Scatchard plots. The range of ^3H -DHE concentrations was 0.2-4.5 nM. Specific binding was defined as binding exceeding that in blanks containing 10^{-5} M phentolamine. Each plot represents one experiment performed in triplicate and replicated 6 times. For control: $K_D = 0.6$ nM, $B_{\max} = 73$ fmol/mg protein. For estrogen treated: $K_D = 0.5$ nM, $B_{\max} = 177$ fmol/mg protein.

IV. Differential effects of nifedipine, verapamil and diltiazem on noradrenaline-induced contractions, adrenergic transmitter release, and alpha-adrenoceptor binding in the female rabbit urethra

To probe for differences in action between Ca^{2+} -entry blockers on smooth muscle and nerve tissue, the effects of nifedipine, verapamil, and diltiazem were studied on NA-induced contractions and electrically evoked release of ^3H -NA in the female rabbit urethra. In addition, possible effects of a series of Ca^{2+} -entry blockers on alpha-adrenoceptors were studied with radioligand binding technique.

Nifedipine, verapamil, and diltiazem all inhibited NA-induced contractions in the rabbit urethra (Fig 7). Nifedipine was approximately 25 times more potent than verapamil and diltiazem. In contrast to verapamil, nifedipine and diltiazem failed to abolish the NA-induced contraction, producing a maximum inhibition of 55 % and 60 %, respectively. The stimulation-induced efflux of ^3H -NA from adrenergic nerve endings in the rabbit urethra was reduced by diltiazem, virtually unaltered by nifedipine, and increased by verapamil (Fig 7).

Amongst the Ca^{2+} -entry blockers examined (nifedipine, nimodipine, felodipine, verapamil and diltiazem), only verapamil inhibited the binding of ^3H -DHE to urethral alpha-adrenoceptors (Fig 7). The inhibition caused by

verapamil appeared to be of the competitive type, as revealed from saturation studies performed in the absence and presence of 3×10^{-7} M verapamil.

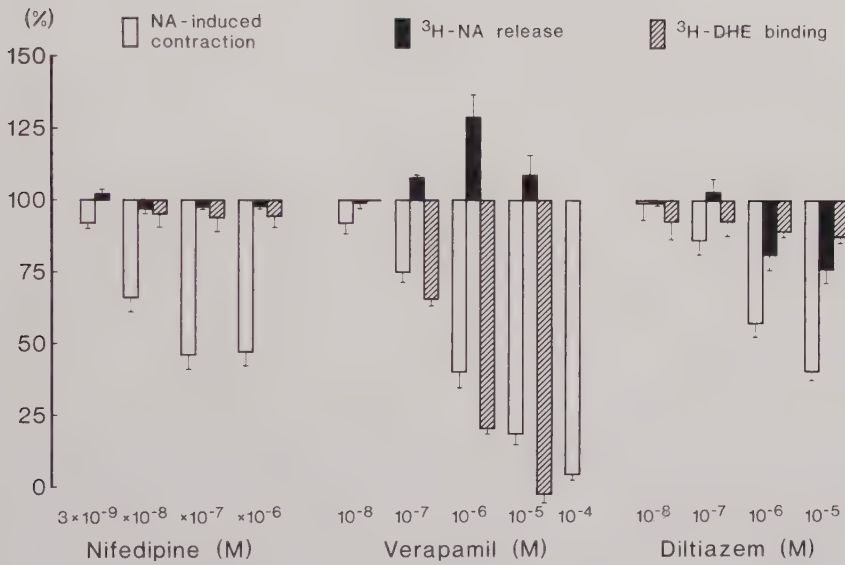


Fig 7. Summary of the results from study IV. Control values were set to 100 %. For further explanation, see study IV.

V. Influence of antimuscarinics on alpha-adrenoceptors in the female rabbit urethra

It has previously been observed in our laboratory that NA-induced contractions were reduced by atropine. We were interested to determine the cause of this depression, and if it was a common feature of antimuscarinics. It has previously been reported that atropine interacts with alpha-adrenoceptor mediated responses (Nedergaard and Schrold 1977, Abraham et al. 1981, Cantor et al. 1983). Therefore, the effects of various antimuscarinics were assessed on NA-induced contractile responses as well as on alpha-adrenoceptor radioligand binding. Some of the results from these studies are summarised in Fig 8. The order of potency for the tertiary antimuscarinic agents atropine, homatropine, and scopolamine on NA-induced contractions and radioligand binding studies, was in both types of experiments atropine > homatropine > scopolamine.

In concentrations of 10^{-5} and 10^{-4} M, the quarternary antimuscarinic agent emeprone inhibited ^3H -DHE binding by 7 % and 56 %, respectively. In contrast, the contractile responses to exogenously administered NA was increased by 19 % and 8 % in the presence of emeprone 10^{-5} and 10^{-4} M, respectively.

No contractile activity per se was found for any of the antimuscarinics tested.

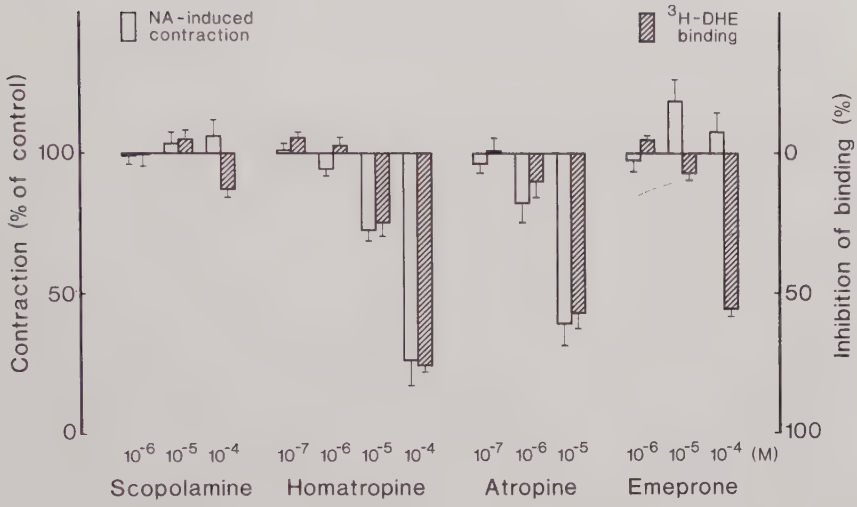


Fig 8. Summary of the results from study V. For explanations, see study V.

GENERAL DISCUSSION

From the results of study (I), it appears that ^3H -DHE fulfills the basic criteria of binding to an alpha-adrenoceptor in the crude membrane preparation obtained from female rabbit bladder base and urethra. This is in accordance with other studies on various peripheral tissues (Williams et al. 1976, Alexander et al. 1978, Tsai and Lefkowitz 1978, Williams and Lefkowitz 1978b). Although ^3H -DHE seems to label alpha-adrenoceptors also in membranes prepared from rat cerebral cortex (Greenberg and Snyder 1977, Greenberg and Snyder 1978), discrepant results have been reported (Davis et al. 1977). In addition ^3H -DHE has been found to bind to dopamine receptors in rat corpus striatum (Greenberg and Snyder 1978) and calf caudate (Tittler et al. 1977).

Levin and Wein (1979), who studied the binding of ^3H -DHE to membranes prepared from male rabbit urinary bladders, found a 4-fold higher density of ^3H -DHE binding sites in the bladder base as compared to the bladder body. A similar predominance of ^3H -DHE binding sites in the female rabbit urethra over bladder body was reported by Johns (1983). Although an extensive characterization of the ^3H -DHE binding sites was not performed in these studies, the findings from functional experiments with these tissues, together with the large body of reports showing binding of ^3H -DHE to alpha-adrenoceptors in peripheral tissues, makes it likely that alpha-adrenoceptors were labelled by ^3H -DHE in these investigations. Hence the results from these studies are

in accordance with the functional predominance of contraction-mediating alpha-adrenoceptors found in the bladder base, as compared to bladder body, where a functional predominance of inhibitory beta-adrenoceptors have been reported (Edvardsen and Setekleiv 1968, Khanna et al. 1981).

Several investigators have reported that the highly selective alpha₁-adrenoceptor antagonist prazosin can give biphasic inhibition curves when various concentrations of this drug is used to inhibit a fixed concentration of ³H-DHE (Miach et al. 1978, Greenslade et al. 1979, Hoffman et al. 1979, Hasegawa and Townley 1982, Ito et al. 1982). Since ³H-DHE is claimed to bind to alpha₁- and alpha₂-adrenoceptors with equal affinity (Hoffman et al. 1979, Hoffman and Lefkowitz 1980), the two phases of the inhibition curve observed for prazosin and rauwolscine (Fig 4) could be assumed to represent displacement of ³H-DHE from the two alpha-adrenoceptor subtypes. The plateaus of the inhibition curves should then represent the proportion of the subtype to which the ligand has selectivity, as compared to the total receptor population occupied by ³H-DHE. This assumption was supported by the finding that the inhibitory effects of 3×10^{-8} M prazosin and 3×10^{-7} M rauwolscine were largely additive. The proportions of alpha₁- and alpha₂-adrenoceptors thus calculated from the plateau of the prazosin inhibition curve was 22 % alpha₁-and 78 % alpha₂-adrenoceptors. The corresponding figures from the rauwolscine inhibition curve was 19 % and 81 %. These

figures were supported by saturation studies using ^3H -prazosin and ^3H -rauwolscine.

Although an extensive series of experiments were not performed with ^3H -prazosin and ^3H -rauwolscine to ensure that these radioligands label an alpha-adrenoceptor in our membrane preparation, preliminary studies indicated that this was the case. The abundance of reports stating that ^3H -prazosin and ^3H -rauwolscine label α_1 - and α_2 -adrenoceptors, respectively, under various assay conditions strengthen this assertion.

In the mechanical activity studies it was found that prazosin was more effective in inhibiting PE-induced contractions than those induced by clonidine. However, the opposite was found when rauwolscine was used to inhibit contractions induced by these agonists. These results suggest that both α_1 - and α_2 -adrenoceptors are present postjunctionally on the female rabbit urethra and are able to mediate contraction.

When the order of potency for clonidine, NA, and PE from the binding studies (II) (clon > NA > PE) was compared with the order of potency in the mechanical activity studies (II) (clon > PE > NA), it was found that PE was more potent than NA in the mechanical activity studies, whereas the opposite was true in the binding experiments. However, in the presence of 10^{-5} M cocaine, the EC_{50} -value for NA was found to be increased about 9-fold as compared to controls (III). Furthermore, the EC_{50} -value for NA was found to be similar in the absence

(II) or presence (III) of 3×10^{-7} M propranolol. In a preliminary set of experiments ($n=2$) the EC_{50} -value for PE increased about 2-fold in the presence of 10^{-5} M cocaine as compared to controls, whereas the EC_{50} -value for clonidine was virtually unaffected by such treatment. Hence it is conceivable that the dissimilar order of potency for clonidine, NA, and PE found in the binding and mechanical activity studies is due to the differences in neuronal uptake between these substances.

Several studies have shown that treatment with estrogens increases the reactivity to catecholamines and other alpha-adrenoceptor agonists of various tissues such as arteries and veins (Altura 1975, Rorie and Muldoon 1979, Colucci et al. 1982), isolated myometrium (Roberts et al. 1981), isolated bladder (Levin et al. 1980b), and urethra (Schreiter et al. 1976, Hodgson et al. 1978). Possible reasons for the increased reactivity to alpha-adrenoceptor stimulation has been suggested to be either an increased affinity to the receptor for alpha-adrenoceptor stimulating drugs (Colucci et al. 1982), or an increased number of alpha-adrenoceptors (Levin et al. 1980b, Roberts et al. 1981).

The present results (III) showed that treatment with estrogen increased the sensitivity of the isolated rabbit urethra to exogenous NA. It was also found that an effect of estrogen on the nerve terminal reuptake mechanism for NA was not a likely reason for this increased sensitivity of the isolated rabbit urethra to

exogenously administered NA, since the increased sensitivity to NA after estrogen treatment remained approximately constant in the presence of 10^{-5} M cocaine (Fig 5).

The radioligand binding studies revealed a 2.4 fold increase in the number of alpha-adrenoceptors in the female rabbit bladder base and urethra after estrogen treatment, with no change in the affinity of ^3H -DHE to the alpha-adrenoceptors. This is in contrast to the findings of Levin et al. (1980b). They reported no change in the alpha-adrenoceptor density in the rabbit bladder base after estrogen treatment. This latter finding is surprising in light of the similar effects of estrogen on the NA content in the urethra and trigone (III), which would implicate similar effects of estrogen on these two structures.

The increase in alpha-adrenoceptor number after estrogen treatment reported in this investigation (III) was found to be largely attributable to a selective increase in the number of α_2 -adrenoceptors as judged from prazosin inhibition curves. The location of these α_2 -adrenoceptors might well be postjunctional as there was no indication in the release experiments, of any change in sensitivity to clonidine or rauwolscine. An estrogen-induced increase in the number of α_2 -adrenoceptors was reported by Hoffman et al. (1981) in the rabbit uterus. However, the main population of these α_2 -adrenoceptors seemed to be located pre-junctionally.

The present results (III) suggest that the increased sensitivity of the isolated female rabbit urethra to exogenously administered NA, at least in part, is attributable to an increase in the number of postjunctional α_2 -adrenoceptors. Possibly this effect of estrogen is one of the factors contributing to the increase in intraurethral pressure (Faber and Heidenreich 1977), and hence to the beneficial effects on stress urinary incontinence seen in women after estrogen treatment (Salmon et al. 1941, Eckerling et al. 1972).

In view of the recent findings of estrogen receptors in the urethra of man (Iosif et al. 1981), and various animal species (Sjögren et al. 1980, Batra and Iosif 1983), one might ask whether the increase in α_2 -adrenoceptors following estrogenization is mediated by mechanisms involving initial binding of estrogen to its own receptor. However, another possible explanation is that the increase in α_2 -adrenoceptor number was secondary to the reduced NA content found in urethra and trigone after estrogen treatment (III). Roberts et al. (1981) studied the time course of estrogen-induced increase in α -adrenoceptor number in the rabbit myometrium. They found that the α -adrenoceptor number did not increase until more than 6 hour after the injection of estrogen, and suggested that macromolecular synthesis may be required for the increase in α -adrenoceptor number in the rabbit myometrium.

Other factors, such as drug exposure and denervation has also been found to alter the alpha-adrenoceptor number (Strittmatter et al. 1977, Arnett and Davis 1979, Bylund et al. 1982, Watanabe et al. 1982). Interestingly, treatments which affect the alpha-adrenoceptor number often seem to involve rather selective changes in α_2 -adrenoceptors (Hoffman et al. 1981, Bylund et al. 1982, Watanabe et al. 1982). Possibly the regulation of the number of α_2 -adrenoceptors is a common feature of cell adaptation to various stimuli.

It is well known that the movement of Ca^{2+} across the cell membrane is an essential regulator of smooth muscle contraction and transmitter release in nerve terminals. The effects of Ca^{2+} -entry blockers, such as nifedipine, verapamil, and diltiazem, have been explained mainly by inhibition of Ca^{2+} -entry into the cell through voltage-dependent Ca^{2+} channels (Bolton 1979, Hagiwara and Byerly 1981, Andersson and Högestätt 1983). However, it has been found that the same organic Ca^{2+} -entry blocker can exhibit rather large differences in potency when interfering with cellular events that are dependent on fluxes of Ca^{2+} in different tissues (Cauvin et al. 1983, Triggle and Swamy 1983), suggesting fundamental differences between Ca^{2+} channels. Indeed, the existence of both voltage-dependent and receptor operated Ca^{2+} channels have been suggested (Bolton 1979). The differences in effects on NA-induced contractions and electrically evoked release of ^3H -NA found between nifedipine, verapamil, and diltiazem (Fig 7), suggest

that different Ca^{2+} -entry blockers might interact with the same Ca^{2+} channel in separate ways. This has been supported both in electrophysiological (Bayer and Ehara 1978, Lee and Tsien 1983) and radioligand binding studies (DePover et al. 1982, Ferry and Glossman 1982).

Although Ca^{2+} -entry blockers are generally considered to interact with membrane channels for Ca^{2+} , and thus influence, for example, the excitation-contraction coupling process at a step subsequent to receptor stimulation, several recent studies suggest additional sites of action for some Ca^{2+} -entry blockers (Church and Zsote $\bar{r}$ 1980, Triggle 1981), including an interaction with alpha-adrenoceptors for verapamil and the related compound D 600. (Fairhurst et al. 1980, Glossman and Hornung 1980, Jim et al. 1981, van Meel et al. 1981, Nayler et al. 1982, Motulsky et al. 1983).

Of the Ca^{2+} -entry blockers tested (IV), only verapamil inhibited ^3H -DHE binding. The ability of verapamil to interact with urethral alpha-adrenoceptors may explain why this drug, in contrast to nifedipine and diltiazem, managed to abolish the NA-induced contractions. In addition, some of the differences in effects between verapamil, and nifedipine and diltiazem on electrically evoked efflux of ^3H -NA might be explained by the alpha-adrenoceptor properties of verapamil.

Also the tertiary antimuscarinic agent atropine has been reported to interact with alpha-adrenoceptors

(Nedergaard and Schrold 1977, Abraham et al. 1981). Atropine was in (V) found to be a potent inhibitor of both NA-induced contractions and ^3H -DHE binding, whereas this ability declined progressively for homatropine and scopolamine, which are structural analogs to atropine (Fig 8). In general, there was a good correlation between the effects of atropine, homatropine, and scopolamine on NA-induced contractions and radioligand binding studies. A 70 fold difference was seen between atropine and scopolamine in inhibiting ^3H -DHE binding. This suggests that the alpha-adrenoceptor blocking properties found for atropine was specific in nature.

The good correlation between the effects of the tertiary antimuscarinic agents atropine, homatropine, and scopolamine on NA-induced contractions and radioligand binding was not seen for the quarternary antimuscarinic agent emeprone. One explanation for this could be that emeprone, in addition to inhibiting ^3H -DHE binding, also blocks the uptake of NA into nerve terminals in the urethral smooth muscle and thus causes deviation supersensitivity (Westfall 1981).

The ability of the Ca^{2+} -entry blocker verapamil and the antimuscarinic agent atropine to interfere with alpha-adrenoceptor mediated events at the level of the alpha-adrenoceptor underline the importance of choosing drugs with specifically desired effects, and moreover using them in appropriate concentrations in order to obtain selective effects of the drugs.

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Demonstration of α -Adrenoceptors in the Rabbit Bladder Base and Urethra with ^3H -Dihydroergocryptine Ligand Binding

By

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Abstract: The purpose of this work was to demonstrate the presence of α -adrenoceptors in a crude membrane preparation made from rabbit bladder base and urethra. This was achieved by radioligand binding studies, using ^3H -dihydro- α -ergocryptine (^3H -DHE) as the radioligand. The specific binding, i.e. the binding that could be inhibited by 10^{-6} M phentolamine, was saturable with 73 fmol ^3H -DHE bound per mg membrane protein. Binding was at steady state after 60 min., and reversible. Rate constants for association and dissociation were $3 \times 10^4\text{ M}^{-1}\text{ min}^{-1}$, and $2 \times 10^{-2}\text{ min}^{-1}$, respectively. A number of compounds were tested for their abilities to compete with ^3H -DHE for the binding sites. The relative affinity of some adrenoceptor agonists was: $(-)$ -adrenaline $> (-)$ -noradrenaline $> (\pm)$ -isoprenaline. Stereoselectivity was shown, since $(-)$ -noradrenaline had 42 times higher affinity than $(+)$ -noradrenaline. Adrenoceptor antagonists inhibited ^3H -DHE binding in the following order of potency: DHE $>$ phentolamine $> (\pm)$ -propranolol. The dissociation constant, K_D , for DHE to the binding sites was estimated in three different ways. The constants were derived from saturation, competition, and kinetic studies, and gave K_D values of 1.1, 1.4 and 0.7 nM, respectively. The results suggest that α -adrenoceptors were labelled by ^3H -DHE in the tissue homogenates.

Key-words: α -Adrenoceptors – radioligand binding – bladder base – urethra – rabbit.

In the bladder base and urethra of man (Ek *et al.* 1977), and several animal species (Edvardsen & Seteklev 1968; Nergårdh 1975), a functional predominance of contraction-mediating α -adrenoceptors has been demonstrated. α -Adrenoceptor-mediated tone is considered to be one of the factors contributing to the maintenance of the intraurethral pressure, and thus, to urinary continence. Until recently these receptors have been characterized by functional methods, using different α -adrenoceptor agonists and antagonists. However, the introduction of radioligand binding techniques has made it possible to identify and characterize numerous receptors for hormones and neurotransmitters (Kahn 1976; Williams & Lefkowitz 1978a). The α -adrenoceptor antagonist ^3H -dihydroergocryptine (^3H -DHE) has been used to identify α -

adrenoceptor binding sites in membrane fractions prepared from various tissues (Williams *et al.* 1976; Greenberg & Snyder 1978; Williams & Lefkowitz 1978b), including the urinary bladder of the rabbit (Levin & Wein 1979).

The functional importance of α -adrenoceptors in the detrusor muscle may be questioned (Nergårdh 1975), but the α -adrenoceptors in the bladder base and the urethra are the targets of, e.g., orally administered α -adrenoceptor agonists used to increase intraurethral pressure in women with stress urinary incontinence (Diokno & Taub 1975; Jonas 1977; Ek *et al.* 1978). The present binding study was performed to demonstrate the presence of α -adrenoceptors in a crude membrane preparation made from rabbit bladder base and urethra. This was achieved by analysis of the saturation of ^3H -

DHE to the α -adrenoceptor sites as well as the inhibition of ^3H -DHE binding by various competing agents. In addition an examination of the kinetics of the ^3H -DHE binding was performed.

Materials and Methods

Membrane preparation. Female albino Danish Land rabbits, weighing 2.4–3.0 kg, were sacrificed by an intravenous air injection through an ear vein. The bladder base and urethra (fig. 1) were rapidly removed, and opened longitudinally. Adherent fat and connective tissue were removed and the mucous membrane scraped off by gentle rubbing with a piece of gauze. Histochemical examination of some specimens (fixed in formaline and stained with haematoxylin-eosin) showed that the mucosa was almost completely removed. The tissue was placed in an isotonic sodium chloride solution, frozen to -80° , and stored up to one month. Preliminary studies showed that frozen urethra gave binding results indistinguishable from those obtained with membranes prepared from fresh tissue.

For each binding experiment, the bladder base and urethra from at least three animals were carefully minced and homogenized with a Polytron PT 10/35 homogenizer (Kinematica GmbH, Kriens/Luzern, Switzerland) for 4×8 sec. (setting 7) in ice-cold buffer containing 50 mM Tris-HCl (pH 7.5 at 23°). The homogenate was filtered through a 0.5 mm nylon mesh (Zürcher Benteltuch Fabrik A.G., Zürich, Switzerland) and centrifuged at $39,000 \times g$ for 20 min. at 4° . The pellet obtained was suspended in fresh buffer and recentrifuged. The subsequent final pellet was suspended in fresh buffer and filtered through a 0.118 mm nylon mesh (Zürcher Benteltuch Fabrik A. G., Zürich, Switzerland). The remaining crude membrane preparation was used in the binding assay. It was shown by histochemical examina-

tion (staining with azan and haematoxylin-eosin) that the tissue trapped on the meshes largely consisted of connective tissue.

Binding assay. Incubation mixtures consisted of 460 μl of the membrane suspension (containing approximately 300 μg protein, corresponding to about 30 mg wet weight of tissue), 20 μl ^3H -DHE solution, and 20 μl redistilled water or solutions of drugs. Tubes in duplicate were incubated in dark environment with shaking at 25° for 60–75 min. (unless otherwise specified). The incubation was terminated by diluting the samples with 1.5 ml of ice-cold buffer, followed by rapid filtration under reduced pressure through Whatman GF/C glass fibre filters pre-soaked with buffer containing 1 mg/ml of bovine serum albumin (BSA), (Serva Feinbiochemia, Heidelberg, W. Germany – Cohn Fract. V., purity $>92\%$). Preliminary experiments revealed that this procedure reduced the binding of free ^3H -DHE to the glass fibre filters (about 50% reduction), without affecting the amount of specific binding. Samples taken from the filtrate showed that no appreciable amounts of membrane protein passed through the filter during the filtration procedure. This was further supported by the finding that filters with smaller pores, although giving higher filter blanks, gave the same amount of specific binding. The filters were washed four times with 5 ml portions of ice-cold buffer. This amount of washing did not reduce the specific binding of ^3H -DHE as compared to smaller washing volumes, but reduced the non-specific binding. The filtration and wash steps were completed in less than 15 sec. After washing, the filters were placed in plastic scintillation vials (Beckman Poly Q, Galway, Ireland) and 1 ml of Protosol (New England Nuclear, Boston, U.S.A.) was added. The vials were heated to 60° for 30 min., and then allowed to cool to room temperature. Subsequently 50 μl glacial acetic acid and 10 ml Econofluor (New England Nuclear, Boston, U.S.A.) were added. Tritium was counted in a LKB RackBeta 1215 liquid scintillation spectrometer with a counting efficiency of about 50% determined with the external standard channels ratio method. Receptor binding refers to the specific binding of the radioligand calculated as the difference between total and non-specific binding. Non-specific binding was determined as the amount of measured radioactivity remaining after inhibition with phentolamine (10^{-5} M), and was found to increase linearly with radioligand concentration up to 3.4 nM, the highest concentration tested. This concentration of phentolamine was chosen since it produced inhibition similar to that of the highest concentration of adrenaline and noradrenaline used. Phentolamine (10^{-5} M) has also been used in similar studies (Williams *et al.* 1976; Strittmatter *et al.* 1977).

In order to determine the concentration of the radioactive ligand in the assay, an aliquot of 20 μl was taken from some tubes, and counted for tritium activity. In saturation experiments, an estimation of free radioligand concentration was made by subtracting total ligand bound from total amount of ligand in the assay. In routine assays approximately 1 nM of ^3H -DHE was

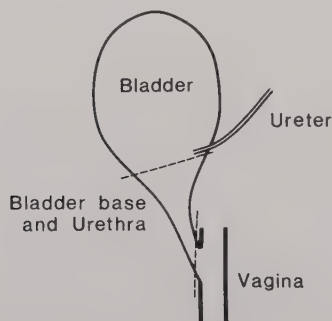


Fig. 1. Schematic drawing of the rabbit bladder and urethra. The part of the rabbit bladder base and urethra used in the binding assays is indicated.

incubated with membranes, and, when appropriate, redistilled water, phentolamine (10^{-5} M) or drugs. The specific binding was at this concentration of ^3H -DHE about 50–60% of total binding. No degradation of ^3H -DHE during incubation could be detected since the specific binding remained constant from 60 to at least 105 min. after initiation of the reaction. At a concentration of 1 nM of ^3H -DHE, approximately 4% of the radioactivity was bound to filters and tissue particles. Even in lower concentrations of ^3H -DHE this fraction did not exceed 10%. This indicates that the reaction could be regarded as being within "zone A" (Straus & Goldstein 1943).

In agonist competition studies, the membranes were pre-incubated at room temperature for 15 min. with the monoamine oxidase inhibitor pargyline in a concentration of 10^{-6} M. All amines were dissolved in water containing EGTA (Ethyleneglycol-bis[β -aminoethyl ether]-N,N'-tetraacetic acid) and ascorbic acid, giving a final concentration of 4×10^{-5} M and 2.8×10^{-4} M, respectively. ^3H -DHE was dissolved in an aqueous solution containing 40% ethanol, giving a final concentration of approximately 1.6% ethanol in the assay tubes. Dihydro- α -ergocryptine mesylate was dissolved in an aqueous solution containing 5 mM HCl and 10% ethanol. Phentolamine, ($\pm$)-propranolol and pargyline were dissolved in water. All solutions were prepared on the day of the experiment.

None of the drugs studied did affect the amount of ^3H -DHE bound to the filters. Neither specific binding nor the "filter blank" was displaced by any solvent used.

Protein content was determined by the method of Lowry *et al.* (1951), using BSA as standard and 50 mM Tris-HCl buffer as blank.

The mean value from duplicate determinations was used for calculations. Linear least square fit of lines to the various plots were used where appropriate. Outliers were checked for by Dixon's gap test (Bliss 1967).

Drugs. ^3H -dihydro- α -ergocryptine (^3H -DHE) (23.0 Ci/mmol) was obtained from New England Nuclear. Dihydro- α -ergocryptine mesylate (Sandoz Ltd., Basel, Switzerland), phentolamine methanesulfonate (Ciba-Geigy AG, Basel, Switzerland), ($\pm$)-propranolol hydrochloride (ICI, Ltd., Macclesfield, Cheshire, U.K.), (+)-noradrenaline hemitartrate (Hoechst), ($\pm$)-isoprenaline hydrochloride (Riker Laboratories), (–)-noradrenaline hydrochloride, pargyline hydrochloride (Aldrich Europe, Beers, Belgium), (–)-adrenaline bitartrate, 5-hydroxytryptamine creatinine sulfate complex (5-HT) and dopamine hydrochloride (Sigma Chemical Co., St. Louis, Mo., U.S.A.).

Chemicals used were of analytical grade. Redistilled water was used for all dilutions.

Results

Saturability of ^3H -DHE binding.

In preliminary experiments, the specific binding of ^3H -DHE to rabbit bladder base and urethral

membranes was found to increase linearly with protein concentration, and to be heat and trypsin sensitive. The binding process was saturable with increasing concentrations of the radioligand (fig. 2). Analysis of the saturation data by a Scatchard plot (Scatchard 1949) yielded a straight line (fig. 3), suggesting the existence of a single population of binding sites. The dissociation constant, K_D , was calculated from the negative reciprocal of the slope of the line, and was found to be 1.1 nM. The intercept of the line with the abscissa revealed the number of binding sites, which was estimated to be 73 fmol/mg membrane protein. No co-operative interactions were found since the analysis of a Hill plot (Bennett 1978) gave a slope (Hill coefficient, n_H) close to unity (fig. 4).

Kinetics of binding.

The association of ^3H -DHE to its binding sites was at equilibrium within 60 min. (fig. 5), and was stable for at least an additional 45 min. period. Since the reaction showed zone A behaviour, the second-order rate constant for association (k_1) could be determined as described by Williams *et al.* (1976), using the equation $k_1 = (k_{obs} - k_2)/[^3\text{H-DHE}]$, where k_{obs} is the observed rate constant for the reversible pseudo-first order reaction and k_2 is the first order rate constant for dissociation. The

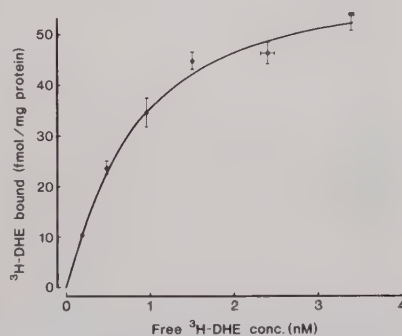


Fig. 2. Specific binding of ^3H -DHE to rabbit bladder base and urethral membranes as a function of free ^3H -DHE concentration. The membranes were incubated with the indicated concentrations of free ^3H -DHE. The data shown are the mean $\pm$ S.E.M. from 5 experiments, each performed in duplicate.

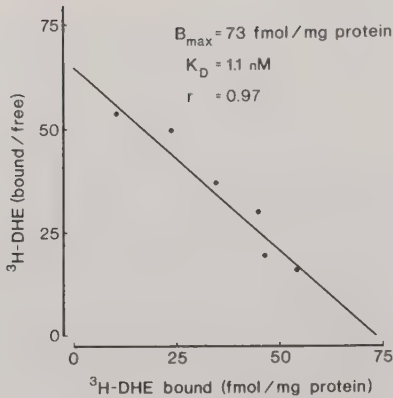


Fig. 3. Scatchard analysis of the data from the same experiments as in fig. 2. The fit of the line was determined by linear regression analysis, where the slope equals $-1/K_D$. The maximum number of binding sites, $B_{\max}$, was estimated from the intercept of the line with the abscissa. The values on the ordinate are given as the ratio bound (fmol/mg) over free (nM) $^3\text{H-DHE}$.

method takes into account the simultaneous association and dissociation of the reversible ligand-receptor complex and the dependence of k_{obs} on the

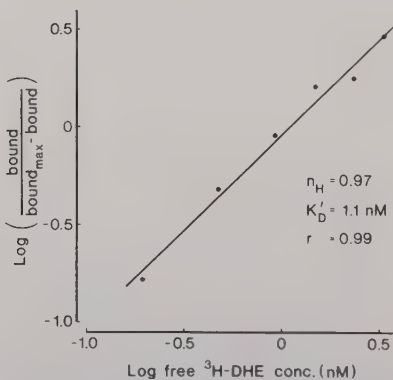


Fig. 4. A Hill plot constructed from the experiments referred to in fig. 2. The value used for maximum number of binding sites, $B_{\max}$, was taken from the Scatchard plot in fig. 3.

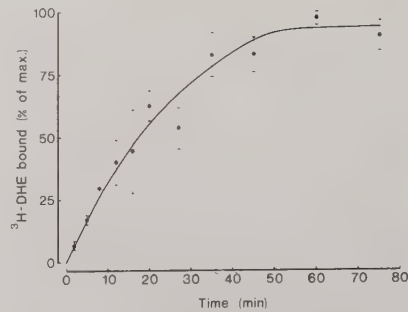


Fig. 5. Specific binding of $^3\text{H-DHE}$ to membranes prepared from rabbit bladder base and urethra as a function of incubation time. Values represent mean $\pm$ S.E.M. of 3 experiments, each conducted in duplicate. An estimate of the observed rate constant for the pseudo-first order reaction could be calculated by plotting $\ln(B_{\text{eq}}/B_{\text{eq}} - B_t)$ versus time, where B_t is the amount bound at time t , and B_{eq} is the amount bound at equilibrium. The slope of the line gives the pseudo-first order rate constant, k_{obs} , which was found to be $5 \times 10^{-2} \text{ min}^{-1}$.

ligand concentration. The rate of dissociation of specifically bound $^3\text{H-DHE}$ was independently determined by incubating membranes to equilibrium at 25° in the presence of the ligand and subsequently adding a large excess of phentolamine (10^{-4} M) to prevent rebinding of dissociated $^3\text{H-DHE}$ (fig. 6). Data from this experiment was used to construct a first order rate plot which has a slope numerically equal to the first order rate constant, k_2 . The value for k_1 was $3 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$, and for k_2 $2 \times 10^{-2} \text{ min}^{-1}$. The K_D value calculated from the ratio k_2/k_1 was found to be approximately 0.7 nM , which is in close agreement with the value of 1.1 nM obtained from saturation experiments.

Specificity of binding.

A number of compounds were tested for their abilities to compete with $^3\text{H-DHE}$ for the binding sites. Since these drugs were considered to interact competitively with $^3\text{H-DHE}$, and since the K_D value for $^3\text{H-DHE}$ is much greater than the receptor concentration, the inhibition constant (K_i) for these compounds could be calculated using the

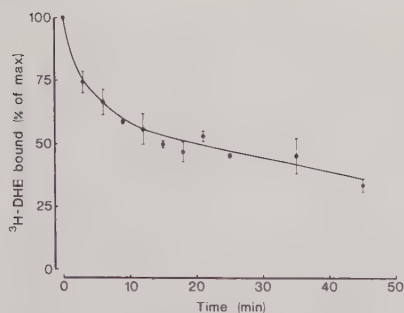


Fig. 6. Dissociation of specifically bound ³H-DHE, from membranes prepared from rabbit bladder base and urethra, as a function of time. Membrane suspensions were preincubated with ³H-DHE. After 60 min. phentolamine was added, giving a final concentration of 10^{-4} M. The time of the addition of phentolamine is defined as $t=0$. Maximum binding (100%) is taken from the amount bound (the mean of phentolamine 10^{-3} and 10^{-4} M used for determination of non-specific binding) just prior to the addition of phentolamine, a straight line could be drawn. The slope of the line gives an estimate of the first order rate constant, k_2 , which was found to be $2 \times 10^{-2} \text{ min}^{-1}$.

equation described by Cheng & Prusoff (1973): $K_i = \text{IC}_{50} / (1 + [\text{DHE}] / K_{\text{DHE}})$, where $[\text{DHE}]$ is the concentration of ³H-DHE in the assay. K_{DHE} is the K_D for ³H-DHE computed from equilibrium binding studies. IC_{50} refers to the concentration of each compound required to inhibit specific binding of radioligand by 50% and was determined graphically for each curve. In separate experiments, phentolamine (10^{-3} M) either in the presence or absence of high concentrations of the tested compounds inhibited ³H-DHE binding to approximately the same extent. Therefore, all drugs used were considered to compete for the same binding sites.

Classical adrenoceptor agonists (fig. 7) competed for the ³H-DHE binding sites in the following manner: (–)-adrenaline > (–)-noradrenaline > (±)-isoprenaline. A pronounced stereoselectivity was found, since (–)-noradrenaline was shown to be 42 times as potent as (+)-noradrenaline in competing with ³H-DHE for the α-adrenoceptor

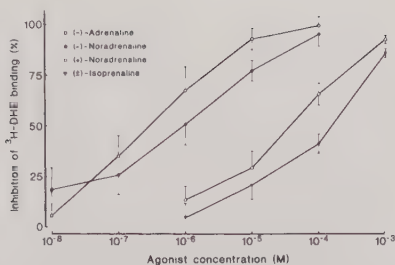


Fig. 7. Inhibition of ³H-DHE binding by various adrenoceptor agonists. Membrane suspensions were incubated with 1 nM ³H-DHE in the presence of the indicated concentrations of the agonists. The amount of ³H-DHE binding inhibited by the various concentrations of the drugs were calculated as per cent of specific binding. Values represent mean $\pm$ S.E.M. of 3 separate experiments, each performed in duplicate.

binding sites (fig. 7). Other amines, such as dopamine and 5-HT, were found to be markedly lower in their affinity for the ³H-DHE-labelled receptor than (–)-noradrenaline (figs. 7 and 8, table 1).

In antagonist competition studies (fig. 9) the α-adrenoceptor antagonists DHE and phentolamine were found to be much more potent than the β-adrenoceptor antagonist (±)-propranolol in inhibiting ³H-DHE-binding. Unlabelled DHE gave a mean K_i value of 1.4 nM which corresponds well with the value of 1.1 nM found in saturation studies

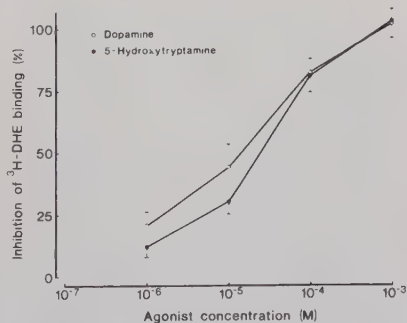


Fig. 8. Inhibition of ³H-DHE binding by dopamine and 5-hydroxytryptamine. Conditions as described in legend to fig. 7.

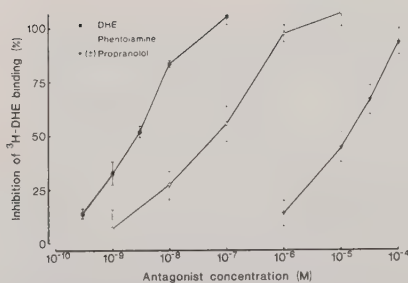


Fig. 9. Inhibition of ^3H -DHE binding by the adrenoceptor antagonists, DHE, phentolamine, and ($\pm$)-propranolol. Conditions as described in legend to fig. 7, except $n=5$ for DHE.

(table 1). This indicates that the tritium labelled form of DHE had similar properties as the native substance.

Discussion

Even if functional studies were not performed in the present series of experiments, the nature of the specific binding of ^3H -DHE to a mixture of rabbit bladder base and urethral membranes indicates that α -adrenoceptors are labelled under the described assay conditions. The specific binding of ^3H -DHE to the receptor sites increased proportionally with increasing protein concentration. The K_D value calculated from the kinetic studies (0.7 nM) was found to be in close agreement with that obtained in saturation studies (1.1 nM). Saturation experiments gave a finite number of receptor sites. When the saturation data were analysed by a Hill plot, a straight line was found with a slope factor (n_H) close to unity, indicating that ^3H -DHE was bound to a homogenous receptor population with no co-operative interactions. In competition studies some well-known adrenoceptor agonists gave the following potency order: ($-$)-adrenaline > ($-$)-noradrenaline > ($\pm$)-isoprenaline. This is the order of potency originally delineated by Ahlquist (1948) to be representative for an interaction with α -adrenoceptors. In addition stereoselectivity was found, since ($-$)-noradrenaline was much more potent in displacing specific ^3H -DHE binding than

Table 1.

Inhibition constants (K_i) for various agonists and antagonists.

Substances	$\log K_i(\text{M})$	mean $K_i(\text{M})$
<i>Agonists</i>		
($-$)-Adrenaline	-6.81 ± 0.37	1.5×10^{-7}
($-$)-Noradrenaline	-6.37 ± 0.37	4.3×10^{-7}
($+$)-Noradrenaline	-4.75 ± 0.13	1.8×10^{-5}
($\pm$)-Isoprenaline	-4.11 ± 0.11	7.8×10^{-5}
Dopamine	-5.22 ± 0.20	6.0×10^{-6}
5-Hydroxytryptamine	-4.90 ± 0.06	1.3×10^{-5}
<i>Antagonists</i>		
Dihydro- α -ergocryptine	-8.84 ± 0.06	1.4×10^{-9}
Phentolamine	-7.52 ± 0.23	3.0×10^{-8}
($\pm$)-Propranolol	-5.19 ± 0.17	6.5×10^{-6}

Values given as mean $\pm$ S.E.M. for $\log K_i$.

Number of determinations, $n=3-5$, each determination performed in duplicate.

its corresponding ($+$)-isomer. Together these findings support the view that an α -adrenoceptor has been labelled by ^3H -DHE in this tissue homogenate.

However, ^3H -DHE may also bind to a different type of receptor, with properties that do not resemble those of "classical" α -adrenoceptors. Davis *et al.* (1977) found that 5-HT was nearly 2 times as potent as ($-$)-noradrenaline, which was about 2 times as potent as dopamine, in displacing ^3H -DHE (8 nM) from a membrane homogenate prepared from rat brain. This effect of 5-HT and dopamine could be avoided by using lower concentrations of ^3H -DHE (0.3–0.4 nM), as shown by Greenberg & Snyder (1977). In contrast to Davis *et al.* (1977) they found that ($-$)-noradrenaline was about 7 times as potent as dopamine, and 59 times more potent than 5-HT. In the present study, ($-$)-noradrenaline was 14 and 30 times more potent than dopamine and 5-HT, respectively. These findings indicate that the problem described by Davis *et al.* (1977) does not apply in the present study.

In their study on the urinary bladder of the rabbit, Levin & Wein (1979) reported a $B_{\max}$ of 78 ± 10 fmol/mg protein, and a K_D of about 7 nM for ^3H -DHE binding to membranes prepared from rabbit bladder base. The total number of α -adrenoceptors in the bladder base reported by these

authors is comparable with that found in the bladder base and urethra in this investigation.

The K_D value was estimated in three different ways in the present study. These were derived from saturation, competition, and kinetic studies, and gave K_D values of 1.1, 1.4, and 0.7 nM, respectively. This is in contrast to the K_D of about 7 nM, reported by Levin & Wein (1979). The reason for this discrepancy is not clear.

Thus, the results from the present study suggest that ^3H -DHE labels an α -adrenoceptor in membranes derived from the bladder base and urethra. Further characterization of the α -adrenoceptors into α_1 - and α_2 -subgroups awaits exploration.

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II

Characterization of the α -adrenoceptors
in the female rabbit urethra

Karl-Erik Andersson, Bengt Larsson & Christer Sjögren

Short title: Urethral α -adrenoceptors

1 A radioligand binding technique for α -adrenoceptors was used to evaluate the proportions of α_1 - and α_2 -adrenoceptors in a crude membrane preparation obtained from the female rabbit bladder base and urethra. In addition isolated urethral rings were studied in vitro in an attempt to determine whether α_1 - and/or α_2 -adrenoceptors are located postjunctionally in the urethral smooth muscle.

2 Inhibition studies of ^3H -dihydroergocryptine binding with the selective α_1 -adrenoceptor antagonist prazosin and the selective α_2 -adrenoceptor antagonist rauwolscine revealed a proportion of approximately 25 % α_1 -adrenoceptors and 75 % α_2 -adrenoceptors. These proportions were confirmed in saturation studies with ^3H -prazosin and ^3H -rauwolscine. The sum of α_1 - and α_2 -adrenoceptors labelled by these selective α_1 - and α_2 -adrenoceptor antagonists was about equal to the number labelled by the non-selective α -adrenoceptor antagonist ^3H -dihydroergocryptine.

3 Noradrenaline as well as the selective α_1 -adrenoceptor agonist phenylephrine and the selective α_2 -adrenoceptor agonist clonidine were able to induce contractions of urethral ring preparations. Prazosin blocked contractions induced by phenylephrine to a greater extent than contractions induced by clonidine. The opposite was true for the inhibitory effect of rauwolscine.

4 In addition to showing the presence of α_1 - and α_2 -adrenoceptor binding sites in the rabbit bladder base and urethra, the results suggest that both of these α -adrenoceptor subtypes are located postjunctionally in the rabbit urethra and mediate contraction.

INTRODUCTION

It is now generally accepted that there are two α -adrenoceptor subtypes, the α_1 - and the α_2 -adrenoceptors. By means of selective agonists and antagonists these α -adrenoceptor subtypes have been characterized in different tissues and species (for reviews, see Langer, 1974; Berthelsen & Pettinger, 1977; Wikberg, 1979; Starke, 1981).

Studies of α -adrenoceptors with radioligand binding technique have shown that various tissues may contain almost exclusively α_1 - or α_2 -adrenoceptors, or a mixture of these (Hoffman, De Lean, Wood, Schocken & Lefkowitz, 1979). Functional studies on isolated vascular smooth muscle have proposed postjunctional α_1 -adrenoceptors in, e.g., the rat portal vein (Ruffolo, Waddell & Yaden, 1981), postjunctional α_2 -adrenoceptors in the feline middle cerebral artery (Skärby, Andersson & Edvinsson, 1983), and both of these subtypes postjunctionally in canine saphenous vein (DeMey & Vanhoutte, 1981) and feline lingual arteries (Skärby, Andersson & Edvinsson, 1983). Moreover, postjunctional

α_1 - and α_2 -adrenoceptors have been suggested to be involved in the pressor responses to α -adrenoceptor agonists in the pithed rat (Timmermans & van Zwieten, 1980) and dog (Constantine, Gunnell & Weeks, 1980), and in the autoperfused hindlimb of the dog (Langer, Massingham, & Shepperson, 1980).

In the urethra, smooth muscle tone (and intraurethral pressure) is believed to be controlled by the sympathetic nervous system via α -adrenoceptors (Donker, Ivanovici & Noach, 1972; Awad & Downie, 1976). It is well established that stimulation of these receptors increases and blockade decreases the intraurethral pressure, and that these effects can be used therapeutically in functional disorders of the lower urinary tract (see, Andersson & Sjögren, 1982). It has been suggested that drugs with selectivity for urethral α -adrenoceptors can be obtained (Gahlin & Sparf, 1978), which makes it of interest to study the α -adrenoceptor subtypes in this region. The aim of the present investigation was therefore to quantify the α_1 - and α_2 -adrenoceptor populations in the female rabbit bladder base and urethra using radioligand binding technique, and to study the functional role of postjunctionally located α_1 - and/or α_2 -adrenoceptors for contraction in the isolated female rabbit urethra.

METHODS

Dissection

Female rabbits of the Danish Land race, (average weight approximately 3 kg) were sacrificed and the bladder and urethra excised en bloc. Starting from the base of the bladder, the urethra was gently dissected free from the vaginal wall down to the urethrovaginal junction and removed.

Radioligand binding studies

Membrane preparation

The rabbit bladder base and urethra were cut open, and the mucosa, periurethral fat and connective tissue removed. The remaining tissue was placed in an isotonic sodium chloride solution, frozen to -80°C , and stored for up to one month. For binding experiments, pooled tissue was carefully minced and homogenized with a Polytron PT 10/35 homogenizer (Kinematica) for 4 x 8 sec (setting 7) in ice cold buffer containing 50 mM Tris-HCl (pH 7.5 at room temperature).

The homogenate was filtered through a 0.5 mm nylon mesh and centrifuged at $39\,000 \times g$ for 20 min at 4°C . The pellet obtained was suspended in fresh buffer and recentrifuged. The subsequent final pellet was resuspended in fresh buffer and filtered through a 0.118 mm nylon mesh and the filtrate was used in the binding experiments.

Binding assay

Tritium labelled dihydro- α -ergocryptine (^3H -DHE), prazosin, and rauwolscine were used.

^3H -DHE assay: The ^3H -DHE binding was performed mainly as described previously (Larsson, 1983). In brief, membranes, ^3H -DHE, and solutions of drugs or their solvents were incubated in triplicate for saturation experiments and in duplicate for inhibition studies in a total volume of 0.5 ml at 25 °C for 60 min. The incubation was terminated by diluting the samples with 1.5 ml of ice cold buffer followed by rapid filtration under reduced pressure through Whatman GF/C glass fibre filters, presoaked with buffer containing 1 mg/ml bovine serum albumin. The filters were rapidly washed four times with 5 ml portions of ice cold buffer.

^3H -Prazosin assay: Membranes, ^3H -prazosin, and redistilled water or phentolamine (10^{-5} M) were incubated in triplicate in a total volume of 0.5 ml at 25 °C for 15 min. The reaction was terminated as described for the ^3H -DHE assay except that three 5 ml washes were used.

^3H -Rauwolscine assay: Membranes, ^3H -rauwolscine, and redistilled water or phentolamine (10^{-5} M) were incubated in triplicate in a total volume of 0.25 ml at 25 °C for 20 min. The reaction was stopped as described for the ^3H -DHE assay except that the samples were diluted with 1 ml of ice cold buffer, and the filters were presoaked with buffer without bovine serum albumin.

The filters were placed in scintillation vials and the tritium content determined in a liquid scintillation spectrometer (LKB RackBeta 1215). The counting efficiency was about 50 % as determined with the external standard channels ratio method.

Preliminary association experiments with the ^3H -ligands showed that steady state was reached within the incubation period for the respective ligand and that binding was stable for at least an additional 15 min period. All reactions were started by the addition of membranes and was continued for 60, 15, and 20 min for ^3H -DHE, ^3H -prazosin, and ^3H -rauwolscine, respectively. Thereafter the filtration procedure was started. This step was completed within 5 - 15 min.

Specific binding was defined as the binding exceeding that in blanks containing 10^{-5} M phentolamine for all tritiated substances. To determine the concentration of radioactive ligand in the assay, an aliquot of 20 μl was taken from some tubes and counted for tritium activity. An estimation of free radioligand concentration in the saturation experiments was obtained from the difference between added and bound ^3H -ligand. Protein content was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin as standard and 50 mM Tris-HCl buffer as blank. Outliers were checked for by Dixon's gap test (Bliss, 1967). The mean value from duplicate or triplicate determinations were used for calculations. Linear least square fit of lines

to the various plots were used where appropriate. K_i -values were determined by the method of Cheng & Prusoff (1973).

Functional studies

The bladder was opened and the trigonal area and the internal urethral orifice identified. Two circular transverse sections, each 2-4 mm long, were taken from the middle and upper parts of the urethra. Each ring-formed preparation was transferred to a temperature controlled organ bath filled with Krebs solution (composition, see below) maintained at 37 °C and bubbled with a mixture of 95 % O_2 and 5 % CO_2 to give a pH of approximately 7.4. The preparation was mounted between two L-shaped steel hooks placed within the lumen of the urethral ring. One hook was connected to a force transducer (Statham FT 03) for registration of isometric tension. The other hook was fixed to a movable unit allowing adjustments of tension. The urethral rings were repeatedly stretched and were then allowed to relax until a stable tension level of approximately 6 mN was obtained.

After an equilibration period of 60 min, the preparations were exposed to 6.2×10^{-5} M noradrenaline (NA), added non-cumulatively, which in separate experiments was found to produce $90 \pm 2\%$ of the maximum response obtained when NA was added cumulatively. Concentration-response curves for NA, phenylephrine (PE), and clonidine were obtained by increasing the drug

concentration cumulatively. The effects of α -adrenoceptor antagonists were tested on a submaximum contractile response produced by a single concentration of agonist. Submaximum concentrations of agonists were used to maintain reproducibility. The antagonist was added when two consecutive, reproducible (less than 10 % variation) responses were achieved; the contact time for the antagonist was 10 - 20 min. The substances were washed out when a maximum response was reached.

Calculations

The results are given as mean $\pm$ SEM. IC_{50} - and EC_{50} -values were determined graphically from the curves. For binding studies, n denotes the number of experiments, whereas for functional studies n states the number of urethral rings tested.

Solutions and drugs

Binding studies

3H -Dihydro- α -ergocryptine was diluted in an aqueous solution containing 40 % ethanol, giving a final concentration of approximately 1.6 % ethanol in the assay tubes. 3H -Rauwolscine was diluted in 1 mM HCl containing 20 % ethanol (approximately 1.6 % ethanol in the assay tubes). 3H -Prazosin was diluted in 1 mM HCl.

Because of difficulties in dissolving prazosin, two stock solutions containing 2.5×10^{-3} and 2.5×10^{-4} M prazosin were made by adding a few drops of methanol and then dissolving prazosin in 1 mM HCl under gentle

heating. In the highest concentration, prazosin had a tendency to precipitate at room temperature and was therefore gently heated just prior to the addition to the assay tubes. In the lower concentration this was not observed. The high concentration stock solution was used for preparing the 2 highest concentrations of prazosin used experimentally; the low concentration stock solution was used for the rest. All dilutions from these stock solutions were made in redistilled water. The effect of NA on ^3H -DHE binding was determined as previously described (Larsson, 1983). All other drugs were dissolved and serially diluted in redistilled water.

Functional studies

The Krebs solution used in the functional studies had the following composition (in mM): NaCl 119, KCl 4.6, CaCl_2 1.5, MgCl_2 1.2, NaHCO_3 15, NaH_2PO_4 1.2, glucose 11. Prazosin was dissolved in ethanol, whereas all other drugs were dissolved in 0.9 % NaCl. Subsequent dilutions of the drugs (including prazosin) were made with 0.9 % NaCl.

^3H -Dihydro- α -ergocryptine (21.9-30.5 Ci/mmol), and ^3H -rauwolscine (84.4 Ci/mmol) were obtained from New England Nuclear, and ^3H -prazosin (20.2 Ci/mmol) from Amersham. Other drugs used were phentolamine methanesulphonate (Ciba-Geigy), rauwolscine HCl (Roth), prazosin HCl (Pfizer), clonidine HCl (Boehringer Ingelheim), (-)-noradrenaline HCl (Aldrich), ($\pm$)-nor-

adrenaline, (-)-phenylephrine (Sigma).

Chemicals used were of analytical grade. Redistilled water was used for preparing all solutions.

RESULTS

Binding studies

When the selective α_1 -adrenoceptor antagonist prazosin, and the selective α_2 -adrenoceptor antagonist rauwolscine were used to inhibit specific ^3H -DHE binding to membranes prepared from the female rabbit bladder base and urethra, biphasic displacement curves were obtained (Figure 1). The prazosin displacement curve reached a plateau at concentrations of 10^{-8} - 10^{-6}M . Using the mean values of the measured inhibition at these concentrations, the level of the plateau was estimated to $22\pm 1\%$ inhibition. Similarly the rauwolscine inhibition curve reached a plateau at concentrations of 3×10^{-7} - 10^{-5}M , corresponding to $81\pm 2\%$ inhibition.

α -Adrenoceptor agonists competed for specific ^3H -DHE binding in the following order of potency; clonidine ($\log K_i = -6.96\pm 0.18$, $n=5$) > (-)-noradrenaline ($\log K_i = -6.36\pm 0.20$, $n=5$) > (-)-phenylephrine ($\log K_i = -5.49\pm 0.09$, $n=5$) (Figure 2).

No additional inhibition of ^3H -DHE binding was obtained when any of these agonists or antagonists were added in

high concentrations together with 10^{-5} M phentolamine, as compared to separate applications of 10^{-5} M phentolamine, suggesting that these substances compete for the same binding sites.

When the displacement of specific ^3H -DHE binding was measured in the presence of 3×10^{-8} M prazosin, an inhibition of binding by 25 ± 4 % ($n=7$) was found. The corresponding value for rauwolscine in a concentration of 3×10^{-7} M was 75 ± 3 % ($n=7$). When these two substances were incubated together in the previously mentioned concentrations (in the same membrane preparation as used for prazosin and rauwolscine displacement), the displacement of specific binding was 93 ± 3 % ($n=7$).

Saturation experiments with ^3H -DHE, ^3H -prazosin, and ^3H -rauwolscine were performed to determine the number of α -adrenoceptors and α_1 - and α_2 -adrenoceptors in the rabbit bladder base and urethra. ^3H -DHE, a non-selective α -adrenoceptor blocker, was found to bind to a saturable number of binding sites amounting to 76 ± 7 fmol/mg protein (Table 1, Figure 3), as revealed by Scatchard plots. When the results from the saturation studies with ^3H -prazosin and ^3H -rauwolscine were treated in a similar manner, finite numbers of receptors were found for both of these substances with B_{max} -values of 19 ± 4 and 51 ± 6 fmol bound/mg protein respectively (Table 1, Figure 3). The Scatchard plots for specific binding of ^3H -DHE, ^3H -prazosin, and ^3H -rauwolscine were all linear, suggesting binding to a single population of binding

sites, i.e. the ligands had an equal affinity to the respective receptors they were bound to.

Functional studies

The non-selective α -adrenoceptor agonist NA as well as the selective α_1 - and α_2 -adrenoceptor agonists PE and clonidine caused contractile responses of the rabbit urethra (Figures 4 and 5). The contractions induced by clonidine developed more slowly than those induced by NA and PE, and relaxation after wash out of the agonist was also slower (Figure 4). The EC_{50} value for NA was 1.2×10^{-5} M ($n=36$), for PE 4.3×10^{-6} M ($n=6$), and for clonidine 2.3×10^{-7} M ($n=6$). PE produced the same maximum contractile force as 6.2×10^{-5} M NA (105 ± 9 %; $n=6$), whereas the maximum force induced by clonidine was lower (73 ± 9 %; $n=6$).

The inhibitory effects of prazosin (10^{-6} M) and rauwolscine (1.5×10^{-6} M) were studied on contractions produced by a single concentration of NA (7.8×10^{-6}). Prazosin depressed the NA-induced contractions by 84 ± 7 % ($n=6$), while rauwolscine inhibited these contractions by 52 ± 4 % ($n=7$).

The inhibitory effects of the same concentrations of rauwolscine and prazosin were also tested on contractions induced by a single concentration of PE (7.9×10^{-6} M). It was then found that rauwolscine caused an inhibition of the contractile response by 34 ± 5 % ($n=11$); prazosin caused a blockade of 89 ± 4 %

(n=6). For clonidine (10^{-6} M)-induced contractions, the corresponding values were 69 ± 5 % (n=5), and 65 ± 8 % (n=5), for prazosin and rauwolscine, respectively. However when a lower concentration of clonidine (10^{-7} M) was used to induce contractions, rauwolscine abolished the responses (n=6), whereas in the presence of prazosin 13 ± 4 % of the contraction was blocked (n=6).

DISCUSSION

It is well known from several studies that the highly selective α_1 -adrenoceptor antagonist prazosin can give biphasic inhibition curves when various concentrations of this drug is used to inhibit the binding of a fixed concentration of ^3H -DHE (Miach, Dausse & Meyer, 1978; Greenslade, Scott, Chasin, Madison & Tobia, 1979; Hoffman, De Lean, Wood, Schocken & Lefkowitz, 1979; Hasegawa & Townley, 1982; Ito, Hoopes, Baum & Roth, 1982). Since ^3H -DHE is claimed to bind to α_1 - and α_2 -adrenoceptors with equal affinity (Hoffman, De Lean, Wood, Schocken & Lefkowitz, 1979; Hoffman & Lefkowitz, 1980), the two phases of the inhibition curve could be assumed to represent displacement of ^3H -DHE from the α_1 - and α_2 -adrenoceptors respectively. The plateau of the inhibition curve will then represent the proportion of the subtype to which the ligand has selectivity, as compared to the total receptor population occupied by the ^3H -ligand. Hoffman, De Lean, Wood, Schocken & Lefkowitz, (1979) concluded that the

discriminating power of prazosin for the α_1 - and α_2 -adrenoceptors in the rabbit uterus "is of such magnitude that the proportion of the subtypes and the dissociation constants of the ligand for each subtype could be graphically estimated from the displacement curve itself. However visual estimates from the yohimbine displacement curve would not provide reliable estimates because of the partial overlap of the two components". Since rauwolscine has been reported to have higher affinity for α_2 -adrenoceptors than yohimbine (Weitzell, Tanaka & Starke, 1979; Langer & Shepperson, 1981; Starke, 1981), we used rauwolscine as a selective α_2 -adrenoceptor antagonist in our studies.

When prazosin was used to inhibit specific ^3H -DHE binding to a crude membrane preparation from the rabbit bladder base and urethra, well marked biphasic displacement curves were obtained. The level of the plateau indicated a proportion of 22 % α_1 -adrenoceptors and accordingly 78 % α_2 -adrenoceptors. The IC_{50} -value estimated graphically from the mean curve for the first phase (α_1 -adrenoceptors) was approximately 2.1×10^{-9} M, and the corresponding value for the second phase (α_2 -adrenoceptors) approximately 3.9×10^{-5} M. Hence it appeared to be a 18 500-fold higher affinity for prazosin to α_1 - than to α_2 -adrenoceptors in these experiments. If the IC_{50} -value for prazosin to the α_1 -adrenoceptor is used in the equation described by Cheng & Prusoff (1973), a K_i -value of approximately 0.9 nM is obtained, which is similar to the K_D -value of 0.4 nM reported for ^3H -prazosin in Table 1.

The plateau of the rauwolscine inhibition curve was not as marked as that for prazosin, indicating that rauwolscine is not as selective for α_2 -adrenoceptors as prazosin is for α_1 -adrenoceptors. The level of the plateau of the rauwolscine inhibition curve revealed a proportion of α_2 -adrenoceptors of 81 %, and hence the proportion of α_1 -adrenoceptors distinguished by rauwolscine was 19 %. This is in good agreement with the values found for prazosin. The discriminating power for the α_1 - and α_2 -adrenoceptor subtypes by rauwolscine was graphically estimated to be approximately 900 times. When the K_i -value for rauwolscine to α_2 -adrenoceptors was calculated in a similar manner to that for prazosin to α_1 -adrenoceptors, a value of 8 nM was obtained. This figure is equal to the K_D -value of 8 nM reported for ^3H -rauwolscine in Table 1.

It could be assumed that prazosin (3×10^{-8} M) and rauwolscine (3×10^{-7} M) displaced different portions of the total α -adrenoceptor population labelled by ^3H -DHE, since the inhibitory effect of these substances was nearly additive under the described assay conditions. These findings support the conclusion that the two phases of the prazosin and rauwolscine inhibition curves represent inhibition of the respective α -adrenoceptor subtype.

For evaluation of the estimated proportions of α_1 - and α_2 -adrenoceptors found in the inhibition studies, saturation experiments with ^3H -DHE, ^3H -prazosin, and ^3H -

rauwolscine were performed. The results from these studies (Table 1) indicated that the sum of receptors determined by ^3H -prazosin and ^3H -rauwolscine were in close agreement with the number found for ^3H -DHE. From the sum of ^3H -prazosin and ^3H -rauwolscine binding sites, ^3H -prazosin constitutes 27 % and ^3H -rauwolscine 73 % of the total number of receptors labelled by these two drugs. These proportions of α_1 - and α_2 -adrenoceptors parallel the previously estimated values from the displacement curves.

When performing radioligand binding studies, the cellular location of the labelled receptors is difficult to settle. Since α -adrenoceptors are known to exist on e.g. adrenergic and cholinergic nerve terminals, and blood vessels, presumably some of these sites have been labelled in these experiments, together with α -adrenoceptors on the smooth muscle. To study the postjunctionally located α -adrenoceptors we therefore examined the contractile effects of the female rabbit urethral smooth muscle, by an in vitro technique.

NA as well as the α_1 -adrenoceptor selective agonist PE and the α_2 -adrenoceptor selective agonist clonidine induced contractions of the urethral smooth muscle preparations. Although these data suggest that both α_1 - and α_2 -adrenoceptors are located postjunctionally and are involved in the contractile response, it is well known that PE and clonidine are not as selective for their respective α -adrenoceptor subtype as the

antagonists prazosin and rauwolscine. Therefore these antagonists were used to inhibit contractions induced by single submaximum concentrations of NA as well as PE and clonidine. It was found that prazosin was able to nearly abolish the contractions induced by the α_1 -adrenoceptor selective agonist PE, while the clonidine (10^{-6} M) induced contractions were less influenced. The inhibitory effect of prazosin on NA-induced contractions was intermediate between that of PE and that of clonidine. However, when rauwolscine was used to inhibit these agonist induced contractions, the potency order was reversed. Furthermore, when a lower, and presumably more "selective" concentration of clonidine (10^{-7} M) was used, rauwolscine abolished the responses, whereas prazosin blocked only about 10 % of these contractions. These results suggest that both α_1 - and α_2 -adrenoceptors are present postjunctionally and mediate contraction.

A strict comparison between binding and functional studies is often difficult to make. In addition to different assay conditions, agonists are frequently found to interact with ^3H -DHE labelled α -adrenoceptors in a way which results in concentration-response curves with shallow slopes. In the present study the agonists NA, PE, and clonidine were all found to interact with the α -adrenoceptors labelled by ^3H -DHE, resulting in flat concentration-response curves. This might be explained by, e.g., different affinities for the agonist to the different α -adrenoceptor subtypes, or possibly to

a negatively cooperated interaction with the receptor. Alternatively these results could be explained by binding of the agonists to a high and a low affinity binding site for one receptor subtype. This has been reported for the α_2 -adrenoceptor (Hoffman, Mullikin-Kilpatrick & Lefkowitz, 1980), but is questioned for the α_1 -adrenoceptor (Stiles, Hoffman, Hubbard, Caron & Lefkowitz, 1983). The interaction between the agonist and the α_2 -adrenoceptor could be affected by guanine nucleotides (Hoffman, Mullikin-Kilpatrick & Lefkowitz, 1980). Possibly the individual cell might be able to regulate the concentration of these nucleotides, and therefore also its reactivity to α -adrenoceptor stimulation. The finding that our membrane homogenate contains about 75 % α_2 -adrenoceptors could well explain the obtained shallow agonist inhibition curves. Furthermore, no β -adrenoceptor blockers or neuronal or extraneuronal NA uptake blockers were used in the functional studies, the presence of which most likely would alter the EC_{50} -values.

In conclusion α -adrenoceptors in a proportion of approximately 25 % α_1 -adrenoceptors and 75 % α_2 -adrenoceptors have been found in a crude membrane preparation from the rabbit bladder base and urethra. It appears that some of these α_1 - and α_2 -adrenoceptors, both are located postjunctionally on the urethral smooth muscle and are able to induce contraction.

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Table 1

Results from Scatchard plots obtained from saturation studies with the indicated tritiated ligands.

<u>Ligand</u>	<u>K_D (nM)</u>	<u>B_{max} (fmol/mg protein)</u>	<u>n</u>
³ H-Dihydro- α - ergocryptine	0.8 $\pm$ 0.1	76 $\pm$ 7	6
³ H-Rauwolscine	8.1 $\pm$ 1.1	51 $\pm$ 6	6
³ H-Prazosin	0.4 $\pm$ 0.1	19 $\pm$ 4	5

Each experiment was performed in triplicate with 5-7 different concentrations of the ligand. n=number of determinations.

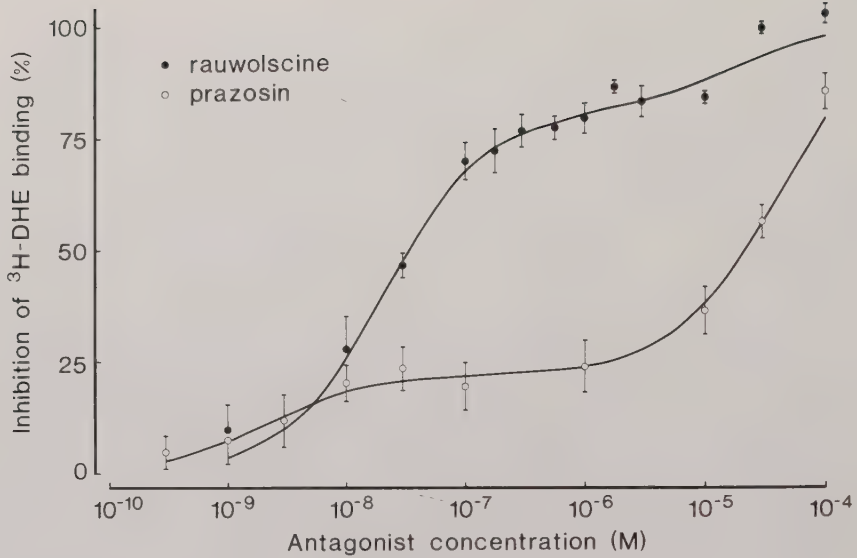


Fig 1. Inhibition of ^3H -DHE binding by prazosin and rauwolscine. Membrane suspensions were incubated with 1 nM ^3H -DHE in the presence of the indicated concentrations of the antagonists. The results are shown as per cent inhibition of specific binding. $n=6$ for both antagonists. Assuming binding to two different binding sites, the lines were drawn according to the equation:

$$\% \text{ inhibition} = \frac{S_1 \times (L)}{K_1 + (L)} + \frac{S_2 \times (L)}{K_2 + (L)}$$

where S_1 and S_2 are the proportions of the respective binding sites, estimated from the plateau of the inhibition curves. K_1 and K_2 are the IC_{50} -values for the inhibition to the respective binding sites. (L) is equal to the antagonist concentration used to inhibit ^3H -DHE binding. For further explanation, see Discussion.

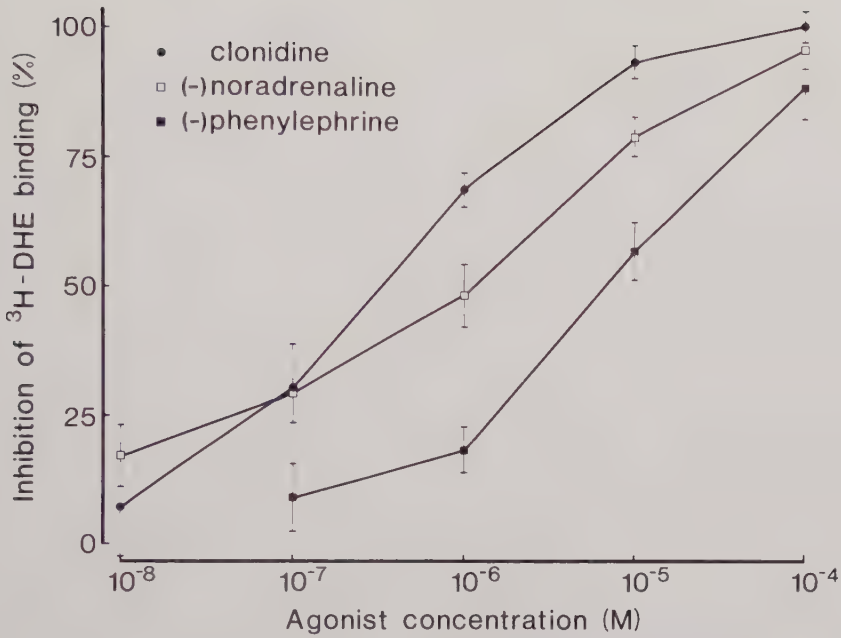


Fig 2. Inhibition of ^3H -DHE binding by various α -adrenoceptor agonists. Membrane suspensions were incubated with 1 nM ^3H -DHE in the presence of the indicated concentrations of the agonists. The results are shown as per cent inhibition of specific binding. $n=5$ for all agonists.

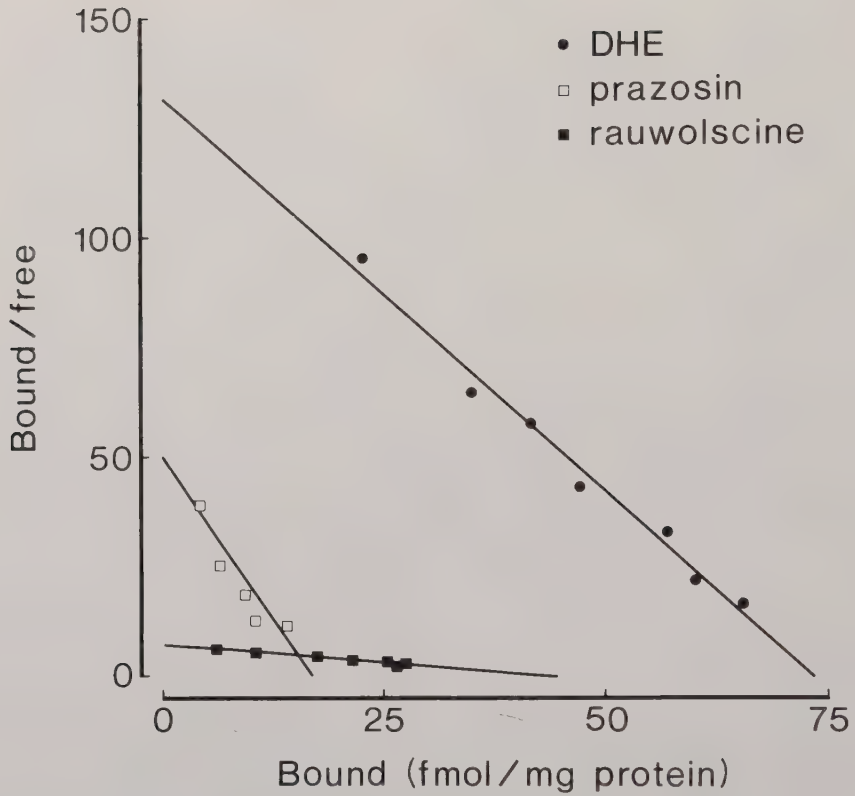


Fig 3. Scatchard plots for ^3H -DHE, ^3H -prazosin and ^3H -rauwolscine. Each plot represents one experiment performed in triplicate and replicated 6 times for ^3H -DHE and ^3H -rauwolscine, and 5 times for ^3H -prazosin. The K_D - and B_{max} -values for these curves were 0.6, 0.4 and 6.3 nM and 71, 17, and 45 fmol/mg protein for ^3H -DHE, ^3H -prazosin and ^3H -rauwolscine, respectively.

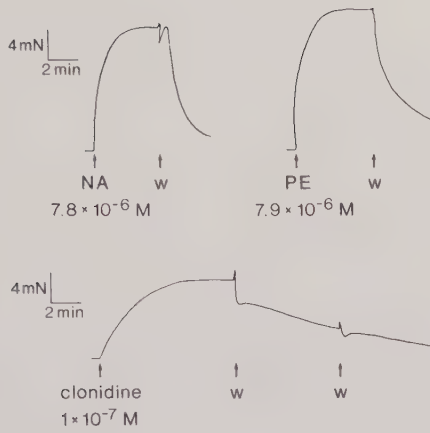


Fig 4. Contractile responses induced by noradrenaline (NA), phenylephrine (PE) and clonidine. w=wash.

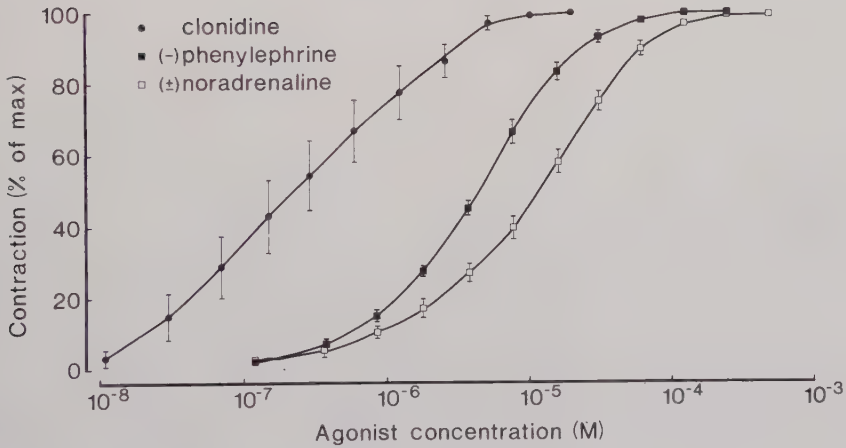


Fig 5. Concentration-response curves for clonidine (n=6), phenylephrine (n=6), and noradrenaline (n=36) added cumulatively to rabbit urethral ring preparations. 100 % denotes the maximum response elicited by each agonist.



Effects of Estradiol on Norepinephrine-Induced Contraction,
Alpha-Adrenoceptor Number and Norepinephrine Content and
Release in the Female Rabbit Urethra

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Running title: Estrogen and alpha-receptors.

ABSTRACT

Larsson, Bengt, Karl-Erik Andersson, Satish Batra, Anders Mattiasson, and Christer Sjögren: Effects of estradiol on norepinephrine induced contraction, alpha-adrenoceptor number and norepinephrine content and release in the female rabbit urethra.

Several studies have revealed that estrogen treatment increases the reactivity to catecholamines of various tissues. In the present study it was found that estrogen treatment caused an increased sensitivity to norepinephrine (NE) of the isolated female rabbit urethra. There was a 3 fold shift to the left of the concentration-response curve for longitudinal tension in perfused urethras and for tension in urethral ring preparations. Possible mechanisms for this increase in sensitivity were investigated by studying radioligand binding to the alpha-adrenoceptors, and by measuring the NE release from and the NE content of the urethras. Using [^3H] dihydro-alpha-ergocryptine ([^3H] DHE) as a marker for alpha-adrenoceptors, a more than 2 fold increase in the receptor number was found after estrogen treatment. This is suggested to be attributable to a selective increase in the number of alpha₂-receptors. No significant change was found in the affinity of [^3H] DHE to the receptor. There were no significant changes in the electrically induced (fractional) release of [^3H] NE.

The total NE content in the urethra was not changed by estrogen treatment. However, if calculated per mg wet weight, the NE content was reduced to half in estrogen treated animals. It is suggested that in the rabbit urethra the estrogen induced increased sensitivity to the contractant effects of NE, at least in part, is attributable to an increase in the number of postjunctional alpha₂-adrenoceptors.

Estrogens are widely used for treatment of stress urinary incontinence in postmenopausal women (Salmon et al., 1941, Eckerling et al., 1972). One reason for this is that estrogens per se have been claimed to increase the intraurethral pressure (Faber and Heidenreich, 1977), another that estrogens were suggested to increase the contractant effects of alpha-adrenoceptor (alpha-receptor) mediated stimulation of urethral smooth muscle (Schreiter et al., 1976). Estrogen treatment has previously been shown to increase the nor-epinephrine (NE) content of rabbit uterus (Sjöberg, 1968). In view of the recent finding of estrogen receptors in the urethra of rat (Sjögren et al., 1980), rabbit (Batra and Iosif, 1983), and man (Iosif et al., 1981), a similar effect of estrogen on the urethra might be found. As the alpha-receptor stimulating drugs mostly used in the treatment of stress urinary incontinence, norephedrine and ephedrine, are considered to exert part of their action by releasing NE, an increased effect of these drugs on the urethra when combined with estrogens (Beisland et al., 1981), may be explained by an increased amount of NE being released and reaching the receptors. On the other hand, estrogens may e.g. also induce cellular changes resulting in increased sensitivity to NE.

In the present study, the effects of estrogen treatment on contractions induced by NE in isolated preparations of female rabbit urethra were investigated. A radioligand binding method was used in order to elucidate whether estrogen treatment caused a change in the receptor characteristics, i.e., in the number of alpha-receptors and/or the affinity of the radioligand for the alpha-receptor. In addition, the electrically induced fractional [^3H] NE release and the content of NE in the tissue were measured.

METHODS

Animals

Mature virgin female albino rabbits of the Danish Land race (2.4 - 3.0 kg) were used, and in experiments with perfused urethras mature virgin female Dutch rabbits (1.5 - 2.4 kg). The animals were divided into two groups.

A. Non-treated animals serving as controls. In separate experiments it was investigated whether or not isolated urethras from oophorectomized rabbits differed in EC_{50} values for NE from non-oophorectomized animals. No such differences were found. Non-oophorectomized rabbits were therefore used as controls.

B. Animals treated with polyestradiol phosphate (ESTRADURIN^R, AB Leo, Sweden) 3 mg i.m. 4 - 5 days before sacrifice.

Experimental procedures

Studies of contractile effects

A. Perfused urethra

After killing of the animals, the bladder and urethra were removed en bloc. Starting from the base of the bladder, the urethra was gently dissected free from the vaginal wall down to the urethrovaginal junction and removed. The intact urethra, including a few mm of the bladder base was mounted in a 30 ml organ bath containing Krebs solution (composition, see below) maintained at 37°C and bubbled with carbogen. The longitudinal tension and resistance to flow were measured simultaneously essentially as described by Persson and Andersson, (1976). A perfusion catheter was inserted near the bladder opening of the urethra, and tied to the bladder base (fig. 1). The catheter was fixed at the bottom of the organ bath. The urethral lumen was perfused with saline at a rate of 3.2 ml/h. The resistance to flow was measured by a pressure transducer (Statham P23DC). The distal part of the preparation was connected to a force transducer (Statham FT03) and isometric longitudinal tension was recorded. The urethra was stretched to approximately 20 mN and was then allowed to relax until a stable basal tone was reached (4-7 mN).

Concentration-response curves for NE were obtained by increasing the drug concentration cumulatively. The initial concentration was about 0.1 μ M and the concentration was increased until maximum longitudinal tension and resistance to flow were obtained. In order to mimic in vivo hormonal environment, 17-beta-estradiol 0.15 ng/ml was present in the bath solution when investigating preparations from group B.

B. Urethral rings

The urethra was dissected out as described above. Rings of tissue (2-3 from each urethra), each 2-4 mm wide, were suspended between 2 L-shaped hooks in 5 ml organ baths containing Krebs solution (composition, see below) maintained at 37°C and bubbled with carbogen. One of the hooks was fixed to a movable unit allowing adjustments of tension, and the other was connected to a force transducer (Statham FT03). Isometric tension was recorded using a Grass (model 7) polygraph. The preparations were stretched and adjusted until a stable basal tone was achieved at 4-6 mN.

The preparations were allowed to equilibrate for 60 min and the response to high potassium Krebs solution (composition, see below) was tested. Only preparations producing a contraction exceeding 10 mN were used for further experiments. After an additional equilibration period of 60 min, NE was added cumulatively to obtain concentration-response curves. All responses to NE were tested in the presence of propranolol 0.3 μ M. The responses to NE were tested in preparations from estrogen-treated animals as well as from controls in the absence and presence of cocaine 10 μ M.

Radioligand binding studies

Membrane preparation

The bladder base and urethra were dissected out and the mucosa and adhering connective tissue removed. The remaining tissue was placed in saline and frozen to -80°C and stored until used (usually within 2 weeks).

For binding experiments, the tissue from several animals was pooled, carefully minced in buffer (50 mM Tris/HCl pH 7.5 at room temperature), and kept on ice when homogenized with a Polytron PT 10/35 homogenizer (Kinematica, Switzerland) for 4 x 8 sec (setting 7).

The homogenate was filtered through a 0.5 mm nylon mesh and centrifuged at $39\,000 \times g$ for 20 min at 4°C . The pellet obtained was suspended in fresh buffer and recentrifuged. The subsequent final pellet was resuspended in fresh buffer and filtered through a 0.118 mm nylon mesh and the filtrate was used in the binding experiments.

Binding assay

Each sample consisted of 460 μl of the crude membrane suspension (corresponding to approximately 30 mg wet weight of tissue, containing approximately 300–400 μg membrane protein), 20 μl [^3H] dihydro-alpha-ergocryptine ([^3H] DHE) solution, and 20 μl water or solutions of drugs. The samples were incubated at 25°C for 60–75 min. The incubation was terminated by diluting the samples with 1.5 ml of ice cold buffer followed by rapid filtration under reduced pressure through Whatman GF/C glass fibre filters, presoaked with buffer containing 1 mg/ml of bovine serum albumin. The filters were rapidly washed with four 5 ml portions of ice cold buffer and subsequently placed in scintillation vials. After the addition of 1 ml Protosol (New England Nuclear), the vials were heated to 60°C for 30 min, and then allowed to cool to room temperature. Glacial acetic acid 50 μl and Econofluor (New England Nuclear) 10 ml was added. Tritium activity was counted in a RackBeta (model 1215, LKB, Sweden) liquid scintillation spectrometer with a counting efficiency of about 50%.

Specific binding was defined as the binding exceeding that in blanks containing 10 μ M phentolamine.

In order to determine the concentration of [3 H] DHE in the assay, an aliquot of 20 μ l was taken from some tubes, and counted for tritium activity. In saturation experiments, an estimation of free [3 H] DHE concentration was obtained from difference between added and bound [3 H] DHE.

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard and 50 mM Tris/HCl buffer as blank. Outliers were checked for by Dixon's gap test (Bliss 1967). For a more detailed description of the various experimental procedures, see Larsson (1983).

NE release studies

The [3 H] NE release studies were performed as described previously (Mattiasson et al., 1983). In brief:

Preparation. The urethra was dissected out as described above. The urethral mucosa, periurethral fat and connective tissue were removed by sharp dissection. A longitudinal piece of the urethral muscle, weighing approximately 0.1 g was cut into eight inter-connecting strips comprising material for two experiments. The preparations were incubated for 45 min with [3 H] NE (1 μ M) in Krebs solution (composition see below) maintained at 37°C and bubbled with carbogen.

Perfusion. Preparations thus obtained were rinsed with Krebs solution in tubes for 3×10 min, followed by continuous perfusion and rinsing for 1 hour in a jacketed perfusion chamber at a flow of 3.8 ml/min (Peristaltic Pump P-3, Pharmacia, Sweden). The temperature of the perfusion chamber (volume 0.4 ml) was thermostatically controlled and set at 37°C . The prewarmed (37°C) perfusate flowed through the chamber from bottom to top.

The preparation was placed in the middle of the chamber between two nets of stainless steel, each in connection with a ringformed electrode placed along the circumference of the chamber. Samples of 5.7 ml perfusate were collected in scintillation vials every 90th second.

Electrical stimulation. A Grass S 48 stimulator delivered 60 seconds trains of square wave pulses of 0.8 ms duration with a current strength of 150 mA (approximately 30 V over the electrodes). The frequency was 10 Hz. The preparations were stimulated at 7, 22, 37 and 52 min after the beginning of the fractional collection, corresponding to sample number 5, 15, 25 and 35 out of totally 45 samples collected in one experiment.

Counting. Ten ml of Lumagel SB (Beckman) was added to each vial. The urethral preparation was dissolved in 2 ml Soluene 350 (Packard) at 60°C for 1 hour before addition of 10 ml scintillation liquid. For counting a liquid scintillation counter, Rack-Beta (model 1215, LKB, Sweden) was used. The efficiency of the counting was approximately 25%.

Calculations. The fractional release was defined as the electrically induced efflux of [^3H] (efflux above baseline level) at one stimulation period in relation to the [^3H] retained by the tissue. The size of each fraction was calculated as the released amount of [^3H] in the first three samples following each stimulation, corresponding to a period of 4.5 min, the stimulation period included. The fractional release at the four stimulation periods, S_I - S_{IV} , were compared in the two groups of rabbits.

NE content studies

Preparation. The urethral smooth muscle was obtained as described previously for release studies. Immediately after preparation the tissue was weighed, placed in tubes, and 0.1 M HClO_4 was added covering the tissue with liquid. The tubes were capped and stored at -80°C .

The time from the killing of the rabbits until the tissue samples were frozen did not exceed 20 min.

The tissues were homogenized the day after dissection. The frozen tissues were transferred to tubes containing 2 ml 0.1 M HClO_4 , with alpha-methyl dopamine (alpha-MDA) 10 ng/ml added as an internal standard. The tubes containing the tissue were kept on ice when homogenized with a Polytron PT 10/35 homogenizer (Kinematica, Switzerland) for 2×15 sec (setting 8). The tubes were centrifuged at $25\,000 \times g$ for 15 min at 4°C . The supernatants were transferred to tubes and stored at -20°C until assayed (usually within a week).

NE assay

The analysis of NE was performed essentially as described by Eriksson and Persson (1982). NE was absorbed on alumina at pH 8.6 and after repeated washes eluted with 0.2 M HClO_4 . A portion of the HClO_4 was injected on a HPLC-column (Nucleosil SA 5 μm from Machery-Nagel), eluted with an acetate/citrate buffer at pH 5.2 with 10% methanol and the catecholamines were finally detected with an electrochemical detector LC-4 (Bioanalytical Systems) equipped with a TL-3 thin-layer cell with a CP-O working electrode.

The relative peak heights of NE/alpha-MDA for the samples were compared with the relative peak heights for NE standards/alpha-MDA processed in the same way as the samples.

Solutions

The Krebs solution had the following composition (mM): NaCl 119, NaH_2PO_4 1.2, KCl 4.6, NaHCO_3 15, MgCl_2 1.2, CaCl_2 1.5, glucose 11. For release and norepinephrine content studies ascorbic acid (0.3 mM) was added. The high potassium solution contained 124 mM KCl and no NaCl but had otherwise the same composition. The solution was bubbled continuously with a mixture of 95% O_2 and 5% CO_2 giving a pH of approximately 7.4 at 37°C. For binding studies the buffer was 50 mM Tris/HCl (pH 7.5 at room temperature).

Drugs

[³H] dihydro-alpha-ergocryptine (21.9-23.0 Ci/mmol), [³H] l-norepinephrine (2.7 Ci/mmol) (New England Nuclear), cocaine HCl (Merck), phentolamine methanesulfonate (Ciba-Geigy), propranolol HCl (ICI), prazosin HCl (Pfizer), rauwolscine HCl (Roth), clonidine HCl (Boehringer Ingelheim), 17-beta-estradiol, d,l-norepinephrine HCl (Sigma), and polyestradiol phosphate (Leo, Sweden).

Statistical analysis

Statistical analysis was performed by means of Student's t-test for paired or unpaired data. $p < 0.05$ was regarded as significant. The results are given as means $\pm$ S.E. unless otherwise stated. EC₅₀ values were determined graphically for each curve.

RESULTS

Studies of contractile effectsA. Perfused urethra.

In the isolated perfused urethra, NE added cumulatively increased resistance to flow as well as longitudinal tension in a concentration related way. The data obtained were used for construction of concentration-response curves for control and estrogen treated rabbits. Estrogen treatment caused a significant shift to the left of the concentration-response curve for NE on longitudinal tension, but not on resistance to flow (figs. 2 A and B).

Table 1 gives the geometric mean EC_{50} value for NE induced changes in longitudinal tension and resistance to flow in the two groups. In the controls, the EC_{50} value for longitudinal tension was approximately 4 times lower than that for resistance to flow ($p < 0.001$). There was a statistically significant decrease in the EC_{50} value for urethral longitudinal tension after estrogen treatment, but not for resistance to flow. The maximum effects of NE on longitudinal tension were for controls 24 ± 4 mN ($n=9$) and for estrogen treated animals 19 ± 5 mN ($n=5$), (difference non-significant). For resistance to flow the values in the two groups were 3190 ± 590 Pa and 3010 ± 150 Pa (difference non-significant).

B. Urethral rings.

In order to investigate whether blockade of the neuronal NE uptake influenced the contractile potency of NE, separate experiments were performed on rings of urethral tissue in the absence and presence of cocaine 10 μ M (fig. 3). In both controls and in estrogen treated animals the EC_{50} values for NE decreased in the presence of cocaine (table 2). The difference in EC_{50} values for NE between the control and estrogen treated groups was 2.8 before and 2.1 after addition of cocaine (table 2). The maximal tension showed no significant change after estrogen treatment (control 0.8 ± 0.1 , $n = 12$, estrogen treated 1.0 ± 0.1 mN/mg wet weight of tissue, $n = 13$).

Radioligand binding studies

The binding of [3 H] DHE to membranes prepared from rabbit bladder base and urethra, was saturable and of high affinity. Analysis of the [3 H] DHE binding data by Scatchard plots (Scatchard 1949) yielded straight lines, indicating the existence of a homogenous population of binding sites, i.e. the ligand had an equal affinity to the receptors it was bound to (fig. 4). Whereas there was no significant change in radioligand affinity (K_D -values) to the receptor, the number of binding sites/mg protein (B_{max}) was more than doubled after estrogen treatment. These data together with the results of Hill plots analysis are shown in Table 3. In addition preliminary experiments on control and estrogen treated animals revealed no difference in the ability of NE to inhibit [3 H] DHE binding.

The ratio between the wet weight (mg) of the tissue, and the amount of protein (mg) obtained in the membrane preparation did not change after estrogen treatment (86 ± 7 before treatment and 84 ± 4 after treatment, $n=6$).

In inhibition studies the selective alpha₁-receptor antagonist prazosin was tested for its ability to compete with the non-selective alpha-receptor antagonist [³H] DHE for the alpha-receptor binding sites. In both controls and animals treated with estrogen, biphasic displacement curves were obtained. The plateau of such a curve is suggested to give an estimate of the proportion of alpha₁-receptors (Andersson *et al.*, 1983)). In the present study the plateau was calculated as the mean of the inhibition values in the concentration interval 0.01 μ M - 1 μ M. Thus, the estimated amounts of alpha₁-receptors were approximately 11% and 21% of the total for treated and non-treated animals, respectively (fig. 5). The number of alpha₂-receptors was calculated by subtracting the estimated number of alpha₁-receptors from the total (fig. 6). As can be seen from figure 6, the estimated number of alpha₁-receptors changed only to a minor degree after estrogen treatment. This suggests that the increase in the number of alpha-receptors was attributed mainly to a selective increase in the number of alpha₂-receptors. Preliminary experiments using [³H] rauwolscine gave further support to such an interpretation of the results.

NE release studies

There was no significant difference in the amount of electrically induced fractional efflux of [^3H] in S_I - S_{IV} between the two groups investigated. In the control group S_I was 1.41 ± 0.07 % (n=12) and in the estrogen treated group the corresponding figure was 1.46 ± 0.13 % (n=12). In the control group, the released amounts of ^3H activity in S_{II} , S_{III} and S_{IV} were 99 ± 2 , 96 ± 2 and 96 ± 2 % of that in S_I . In the estrogen treated group the corresponding values were 95 ± 2 , 94 ± 3 and 90 ± 3 % (n = 12).

In a separate series of experiments the effects on the [^3H] release of clonidine $1 \mu\text{M}$ (n = 5) and rauwolscine $0.1 \mu\text{M}$ (n = 4) were tested before and after estrogen treatment. In comparison with control (100%) clonidine significantly ($p < 0.001$) decreased the release of [^3H] to 68 ± 3 % in untreated and to 71 ± 4 % in estrogen treated animals. Rauwolscine significantly ($p < 0.001$) increased the release of [^3H] to 191 ± 14 % in untreated and to 191 ± 16 % in estrogen treated animals (differences between treated and untreated animals were non-significant).

NE content studies

The total content of NE in the urethra was not changed by estrogen treatment (table 4). However, in the estrogen treated group the wet weight of the urethra was almost doubled (the ratio wet weight/dry weight was the same in estrogen treated animals and controls). Thus, the NE content per mg wet weight of the organ was about halved after estrogen treatment. The NE content per mg wet weight in the trigone was also approximately halved with estrogen treatment (table 4).

DISCUSSION

It is well known from several studies that treatment with estrogens increases the reactivity to catecholamines and other alpha-receptor agonists of various tissues such as arteries and veins (Altura, 1975, Rorie and Muldoon, 1977, Colucci et al., 1982), isolated myometrium (Roberts et al., 1981), and isolated bladder (Levin et al., 1980), and urethra (Hodgson et al., 1978). The present results showed that treatment with estrogen increased the sensitivity of the isolated rabbit urethra to exogenous NE. One reason for this enhanced sensitivity may be interference with the processes in the tissues which inactivate NE so that drug which normally goes to "sites of loss" is deviated to the receptors ("deviation supersensitivity", Westfall, 1981). Sjöberg (1968) showed that estrogen treatment increased the NE content of rabbit uterus. If estrogen causes an increase of the NE content in the human urethra, it is tempting to speculate that the increase in intraurethral pressure, produced by estrogens when given alone to women (Faber and Heidenreich, 1977, Rud, 1980), or together with indirectly acting alpha-receptor agonists such as ephedrine or norephedrine (Beisland, 1981), will be caused by an increased release of NE. However, the present results clearly showed that the total NE content of the rabbit urethra did not change, but rather that when calculated per mg wet weight it was halved. Similar changes were found in the trigone. Further, the fractional release of NE did not change with estrogen treatment. These results are in line with that of Bengtsson (1978) who found that estrogen treatment rather decreased the amount of [^3H] NE being released by potassium in the rat uterus.

The finding that addition of cocaine to urethral rings from controls and estrogen treated animals markedly increased the sensitivity to NE shows that an uptake mechanism is of importance for the inactivation of NE in the rabbit urethra. Estrogen treatment decreased the EC_{50} value for NE 2.8 times, and it cannot be excluded that this increased sensitivity to NE may be caused by inhibition of the amine pump. On the other hand, the difference in EC_{50} value for NE between the control and estrogen groups persisted also in the presence of cocaine which does not favour such an explanation.

In the perfused non-estrogen treated urethras, the EC_{50} value for NE on longitudinal tension was significantly lower than that on resistance to flow. Resistance to flow reflects mainly circular muscle activity (Persson and Andersson, 1976). In the perfused urethras the EC_{50} value for NE on resistance to flow was similar to that found in the urethral rings (which also reflect circular muscle activity). This finding suggests that the longitudinal muscle is more sensitive to NE than the circular muscle. After estrogen treatment, the sensitivity to NE of the longitudinal muscle increased significantly. When recording circular muscle activity after estrogen treatment, the EC_{50} value for NE was decreased significantly only in the urethral rings. This may be attributed to the methods of recording rather than to less pronounced effects of estrogen on circular muscle. The technique of recording intraluminal perfusion pressure may be the more physiological, but isometric tension recordings the more sensitive way to register changes in concentration - effect relations (Moulds, 1983).

Supersensitivity attributed to an increased responsiveness of the smooth muscle cells ("non-deviation supersensitivity", Westfall, 1981) may be caused by e.g. an increase in the number of receptors in the muscle. Roberts et al., (1981) found a several-fold increase of the sensitivity to NE (4.5 times) and methoxamine (9 times), but not to acetylcholine, in isolated uterine strips after estradiol treatment. This was suggested to be attributable to an increase in alpha-receptor number. Levin et al., (1980) showed on isolated rabbit urinary bladder that estrogen treatment increased the maximum contractile response to methoxamine, but the ED₅₀ value was unchanged. They also found increased maximum responses to betanechol and ATP, but not to high potassium. In parallel with the increases in the responses they found increases in the number of alpha- and muscarinic receptors and concluded that the augmented responses to alpha- and muscarinic receptor stimulation were a result of the increased receptor densities. Colucci et al., (1982) found that in vascular smooth muscle from male rats, estrogen treatment caused an increase of sensitivity to catecholamine-induced contraction. This was attributed to an increase in the receptor affinity for catecholamines, as there were no increase in the alpha-receptor number (alpha₁ receptors). Hodgson et al., (1978) reported that the sensitivity to phenylephrine of the isolated rabbit urethra increased after estradiol treatment (a 3-fold decrease of the ED₅₀ value for contraction of both annular and longitudinal segments). In the present study on the bladder base and urethra, a 2.7 fold increase in the number of alpha₂-receptors without a change in the affinity for [³H]DHE to the alpha-receptors was

found. This finding may well contribute to the increased sensitivity of the urethral muscle to NE. About 75% of the alpha-receptors of the rabbit bladder base and urethra were found to be of alpha₂-type, and selective alpha₂-receptor agonists have pronounced contractile effects on the isolated rabbit urethra (Andersson et al., 1983). The localization of these receptors might well be post-junctional as there were no indications, in the release experiments, of any changes in fractional release between urethras from treated or non-treated animals neither in the absence nor presence of clonidine or rauwolscine. An estrogen-induced increase in the number of alpha₂-receptors was reported by Hoffman et al., (1981) in the rabbit uterus. However, the main population of these alpha₂-receptors seemed to be located prejunctionally.

In rabbit myometrium Roberts et al. (1981) found that estrogen caused a time- and dose-dependent increase in alpha-receptor concentration and suggested that a macromolecular synthesis was required for this increase. It is noteworthy that the increase in alpha-receptors found in the present study is not simply due to an increase in cell size or cell number, but a real increase in terms of the density of receptors per unit tissue mass or mg membrane protein. This is in contrast to the effects of estrogen on rat uterine estrogen receptors which keeps up rather well with tissue growth, but do not increase per unit mass or tissue protein (Katzenellenbogen et al., 1977, Manni et al., 1981). Consequently, an intriguing question is whether the increase in alpha₂-receptors

following estrogenization is mediated by mechanisms involving initial binding of estrogen to its own receptors, since high affinity estrogen receptors have recently been demonstrated in both the cytosol and nuclei of the urethral smooth muscle cell (Sjögren et al., 1980, Iosif et al. 1981, Batra and Iosif, 1983). Although this is not an unlikely possibility since estrogenization in target tissues leads to an overall increase in the synthetic activity of the tissue, data on the temporal effects of estradiol on estrogen receptors, and on alpha-receptors would be required to answer this question.

In conclusion, the present study showed that estrogen treatment increases the sensitivity to NE in rabbit urethral smooth muscle. The NE content per mg wet weight was halved, but the ability to fractionally release NE from adrenergic nerves was not changed. Estrogen treatment caused a conspicuous increase in the number of alpha-receptors, but the affinity of [³H] DHE to these receptors was unchanged. Whether such changes can be obtained also in the human female urethra remains to be established.

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TABLE 1

Contractile effects of NE on perfused urethras.

Log EC_{50} values (means $\pm$ S.E.) for NE (M) on isolated urethras from controls and from estrogen treated rabbits. The antilog of the mean value (μ M) is given within brackets.

Group	Longitudinal tension	Resistance to flow
Control (n=9)	-5.42 ± 0.06 (= 3.8)	-4.81 ± 0.07 (= 15)
Estrogen (n=5)	-5.89 ± 0.09 (= 1.3)	-4.93 ± 0.08 (= 12)
	p < 0.001	N.S.

TABLE 2

Contractile effects of NE on urethral rings.

Log EC_{50} values (mean $\pm$ S.E.) for NE (M) on urethral ring preparations from controls and from estrogen treated animals. The antilog of the mean value (μ M) is given within brackets.

	No cocaine	Cocaine 10 μ M
Control (n=8 and 10, respectively)	-4.84 ± 0.06 (= 14)	-5.79 ± 0.10 (= 1.6)
Estrogen (n=7 and 9, respectively)	-5.30 ± 0.14 (= 5.0)	-6.12 ± 0.10 (= 0.76)
	p < 0.01	p < 0.05

TABLE 3

Results from Scatchard and Hill plots.

Each experiment was performed with 7 different concentrations of [^3H]DHE (ranging between 0.2 - 4.5 nM), and analysed separately. For each concentration of the radiolabelled ligand triplicate determinations were performed, and means used for calculations. Specific binding was defined as binding exceeding that in blanks containing 10 μM phentolamine. Values are given as mean $\pm$ S.E.

	Scatchard plots			Hill plots		
	K_D (nM)	$B_{\max}$ (fmol/mg prot)	r	K_D (nM)	n_H	r
Control (n=6)	0.7 $\pm$ 0.1	78 $\pm$ 9	0.90 $\pm$ 0.03	0.7 $\pm$ 0.07	0.96 $\pm$ 0.07	0.93 $\pm$ 0.03
Estrogen (n=6)	0.6 $\pm$ 0.1	184 $\pm$ 13	0.97 $\pm$ 0.01	0.6 $\pm$ 0.1	0.95 $\pm$ 0.01	0.98 $\pm$ 0.01
	N.S.	p < 0.001				

TABLE 4

NE content (ng) and wet weight (mg) of the urethra and trigone from control and from estrogen treated animals.

Values are given as mean $\pm$ S.E.

	NE content	Wet weight	Ratio
Urethra: Control (n=6)	123 $\pm$ 8	114 $\pm$ 10	1.10 $\pm$ 0.08
Estrogen (n=6)	125 $\pm$ 18	202 $\pm$ 17	0.63 $\pm$ 0.10 p < 0.01
Trigone: Control (n=6)	35 $\pm$ 4	43 $\pm$ 5	0.81 $\pm$ 0.05
Estrogen (n=6)	.25 $\pm$ 4	54 $\pm$ 8	0.46 $\pm$ 0.04 p < 0.001

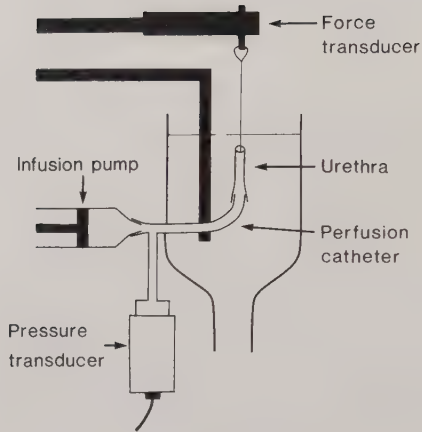


Fig. 1.

Schematic drawing of the device for recording of longitudinal tension and resistance to flow in the isolated rabbit urethra. The perfusion catheter was fixed, and the force transducer movable for initial stretching of the preparation.

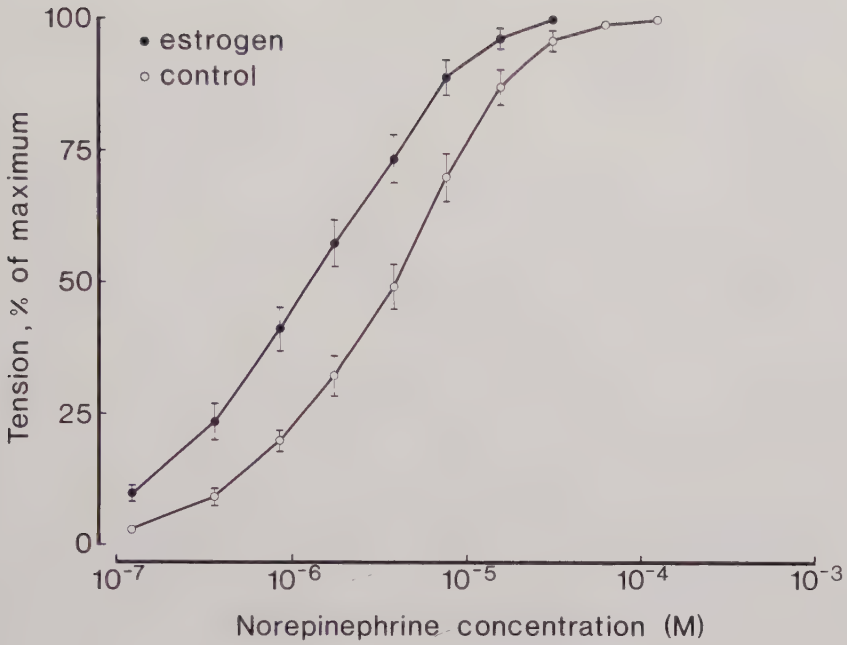


Fig. 2 A.

Concentration-response curves for norepinephrine on the longitudinal tension of isolated rabbit urethra. For number of determinations and EC_{50} -values see Table 1.

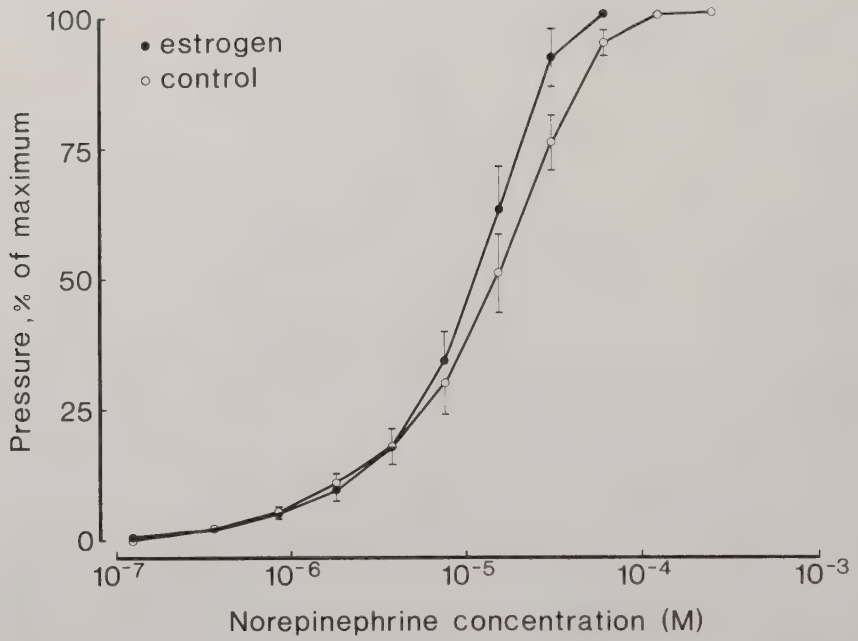


Fig. 2 B.

Concentration-response curves for norepinephrine on the resistance to flow of isolated rabbit urethra. For number of determinations and EC_{50} -values, see Table 1.

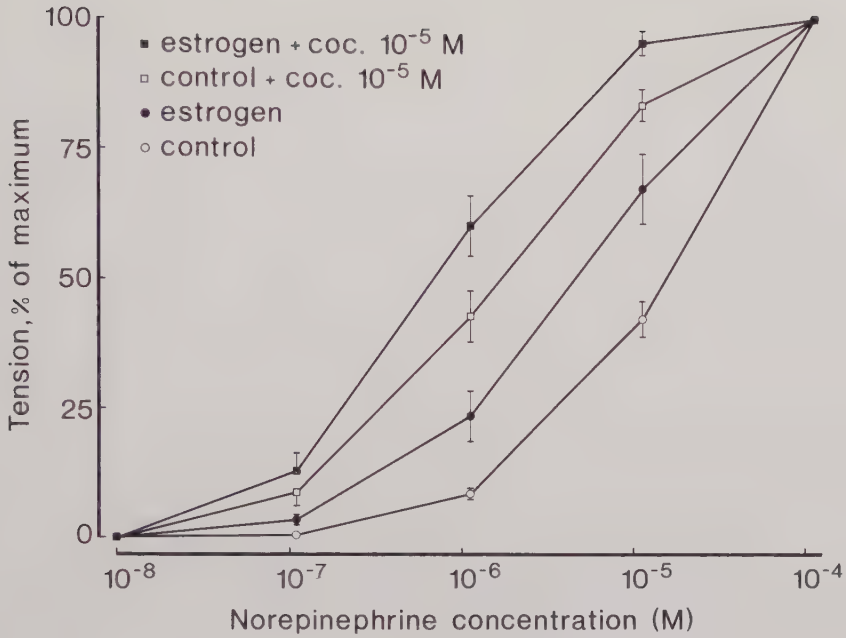


Fig. 3.

Concentration-response curves for norepinephrine on ring preparations from rabbit urethra in the presence or absence of $10\ \mu\text{M}$ cocaine. All responses were recorded in the presence of $0.3\ \mu\text{M}$ propranolol. No further contraction was recorded with $1\ \text{mM}$ norepinephrine in any of the groups.

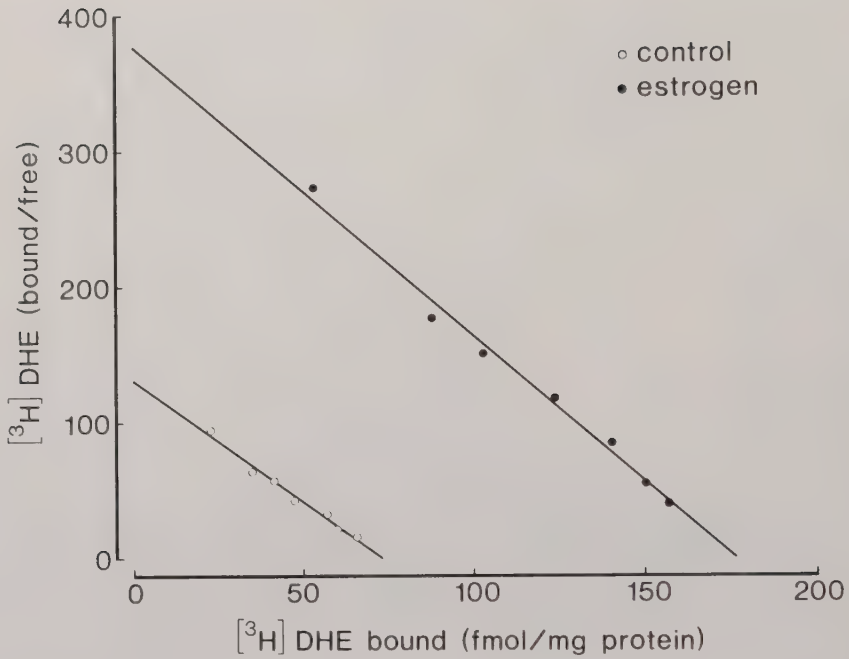


Fig. 4.

Scatchard plots for control and estrogen treated rabbits.

The range of [^3H] DHE concentrations was 0.2–4.5 nM. Specific binding was defined as binding exceeding that in blanks containing 10 μM phentolamine. Each plot represents one experiment performed in triplicate and replicated 6 times. For control: $K_D = 0.6$ nM, $B_{\text{max}} = 73$ fmol/mg protein, $r = 0.99$. For estrogen treated: $K_D = 0.5$ nM, $B_{\text{max}} = 177$ fmol/mg protein, $r = 0.99$.

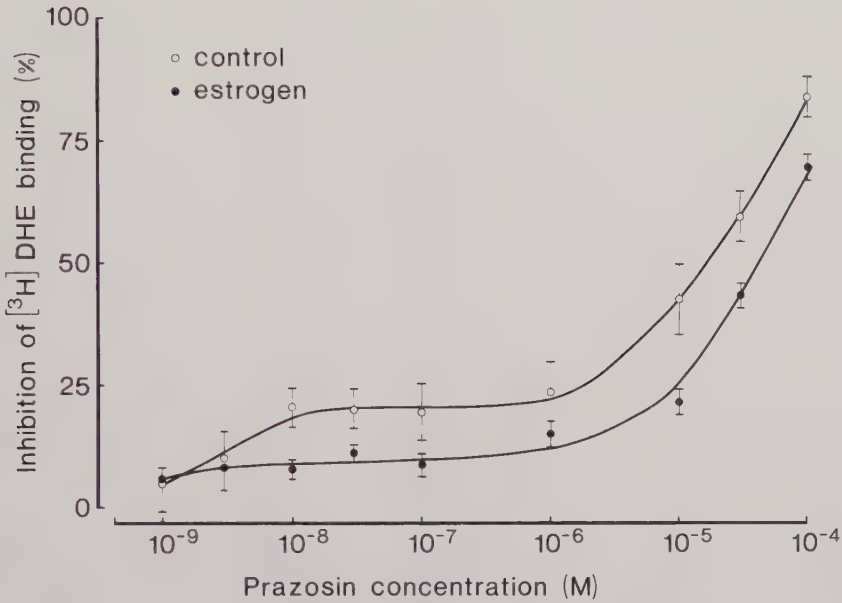


Fig. 5.

Inhibition of [³H]DHE binding by the selective α_1 -receptor antagonist prazosin. Membrane suspensions were incubated with [³H]DHE (1 nM) in the presence of the indicated concentrations of prazosin. The data are shown as percent inhibition of specific binding, which was defined as binding exceeding that in blanks containing 10 μ M phentolamine. Number of determinations, $n = 6$ for controls, and $n = 5$ for estrogen treated rabbits. Each determination was performed in duplicate, and the mean was used for calculations.

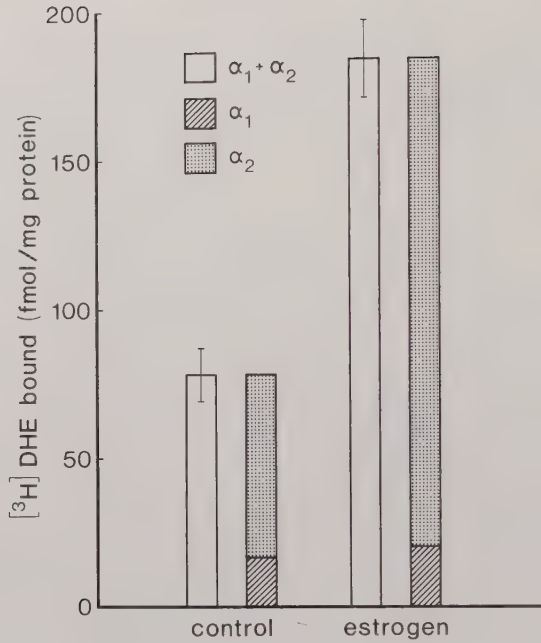


Fig. 6.

The proportion of α_1 - (α_1) and α_2 - (α_2)-receptors in the membrane preparation from rabbit bladder base and urethra. The total number of α -receptors/mg protein in control and estrogen treated animals was taken from Table 3. The values representing the amount of α_1 -receptors were estimated from the plateaus of the inhibition curves (Fig. 5), where prazosin was used as an inhibitor of specific $[^3\text{H}]$ DHE binding. The number of α_2 -receptors were then obtained by subtracting the number of α_1 -receptors from the total number of receptors.

IV

Differential effects of nifedipine, verapamil, and diltiazem on noradrenaline-induced contractions, adrenergic transmitter release, and alpha-adrenoceptor binding in the female rabbit urethra

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Running title: Ca^{2+} -entry blockers, alpha-adrenoceptors, and the rabbit urethra.

Key words: Ca^{2+} -entry blockers, alpha-adrenoceptors, smooth muscle, noradrenaline release, rabbit urethra

ABSTRACT

In order to study differences in action between "Ca²⁺-entry blockers" on smooth muscle and peripheral nerves, the effects of nifedipine, verapamil, and diltiazem on noradrenaline (NA)-induced contractions and electrically evoked release of ³H-NA were investigated in the female rabbit urethra. In addition, possible influences of Ca²⁺-entry blockers on alpha-adrenoceptors were studied with radioligand binding technique. Exposure to Ca²⁺-free medium completely abolished the contractile response to 1 μM NA in the rabbit urethra, indicating that the contraction was entirely dependent on influx of extracellular Ca²⁺. The Ca²⁺-entry blockers inhibited the NA-induced contractions in the following order of potency: nifedipine > verapamil > diltiazem. In contrast to nifedipine and diltiazem, which produced a maximum inhibition of between 50 and 60 %, verapamil was able to abolish the contractile responses to NA. The electrically evoked efflux of ³H-NA was decreased by diltiazem and increased by verapamil, whereas nifedipine failed to alter the ³H-NA efflux. Only verapamil was effective in inhibiting specific ³H-DHE binding to a crude membrane preparation of the rabbit bladder base and urethra, and the inhibition appeared to be of the competitive type. It is suggested that the effects of verapamil on electrically evoked efflux of ³H-NA and on NA-induced contractions can be partly explained by blockade of pre- and post-junctional alpha-adrenoceptors. The failure of nifedipine and diltiazem

to abolish the NA-induced contraction might indicate the existence of different Ca^{2+} -entry pathways in urethral smooth muscle.

INTRODUCTION

Organic " Ca^{2+} -entry blockers", such as nifedipine, verapamil, and diltiazem, are powerful smooth muscle relaxants (Fleckenstein, 1977), which are currently being evaluated in the treatment of a number of cardiovascular disorders, including vasospastic angina and arterial hypertension (Antman et al., 1980; Ellrodt et al., 1980; Henry, 1980; Stone et al., 1980). Their effects have been explained mainly by inhibition of Ca^{2+} -entry through voltage-dependent Ca^{2+} channels in the muscle membrane (Nayler and Poole-Wilson, 1981; Andersson and Högestätt, 1983). However, Ca^{2+} -entry blockers display selectivity for certain voltage-dependent, Ca^{2+} channel-mediated processes. For example, excitation-contraction coupling in cardiac and smooth muscle is particularly sensitive to Ca^{2+} -entry blockers, whereas stimulation-secretion coupling in vertebrate neurons appears to be relatively resistant to most of these drugs (Fleckenstein, 1977; Hagiwara and Byerly, 1981), suggesting fundamental differences between Ca^{2+} channels (Hagiwara and Byerly, 1981). Furthermore, different Ca^{2+} -entry blockers interact with the same Ca^{2+} channel in separate ways (Bayer and Ehara, 1978), suggesting differences also in the site or mode of interaction with the Ca^{2+} channel between the drugs.

Recently, some Ca^{2+} -entry blockers have been used as markers of Ca^{2+} channels in radioligand binding studies. Thus, the 1,4-dihydropyridines ^3H -nitrendipine, ^3H -nimodipine, and ^3H -nifedipine were found to bind with high affinity to cardiac, smooth muscle, and brain membranes (DePover et al., 1982; Ehlert et al., 1982; Ferry and Glossmann, 1982; Holck et al., 1982; Marangos et al., 1982). Based on the ability to influence the binding of ^3H -nimodipine, Glossmann et al. (1982) suggested that Ca^{2+} -entry blockers can be subdivided into three major groups. According to their classification system the structurally dissimilar Ca^{2+} -entry blockers nifedipine, verapamil, and diltiazem belong to different subgroups.

In order to further study differences in action between Ca^{2+} -entry blockers on smooth muscle and peripheral nerves, the effects of nifedipine, verapamil, and diltiazem on noradrenaline (NA)-induced contractions and electrically evoked release of ^3H -NA were investigated in the female rabbit urethra. Urethral preparations of various species have been found to be richly innervated by adrenergic nerves (Owman et al., 1971; Benson et al., 1976) and respond with strong contractions to transmural electrical stimulation. Some Ca^{2+} -entry blockers have been shown to interact with various membrane receptors, such as alpha-adrenoceptors, muscarine receptors, and opiate receptors in a variety of tissues (Blackmore et al., 1979; Fairhurst et al., 1980; Glossmann and Hornung, 1980; Jim et al., 1981; van

Meel et al., 1981; Barnathan et al., 1982). Therefore, we also examined the effects of various Ca^{2+} -entry blockers on alpha-adrenoceptor binding to a crude membrane preparation of the female rabbit bladder base and urethra.

MATERIALS AND METHODS

Dissection

Female rabbits of the Danish Land race, weighing 2.4 to 3.4 kg, were sacrificed by an injection of air into an ear vein. After opening of the abdominal cavity, the bladder and urethra were excised en bloc and periurethral fat and connective tissue removed by combined blunt and sharp dissection.

Recording of mechanical activity

Ring preparations of the rabbit urethra (3 to 4 segments per urethra), approximately 2 mm wide, were suspended between two L-shaped specimen holders (diameter = 1 mm) in a small volume muscle bath (5 ml), containing "standard" Krebs solution (37 °C). The standard Krebs solution was composed of (in mM): NaCl 119, KCl 4.6, CaCl_2 1.5, MgCl_2 1.2, NaHCO_3 15, NaH_2PO_4 1.2, glucose 11, and was continuously bubbled with 95 % O_2 and 5 % CO_2 , resulting in a pH of approximately 7.4. Tension was measured "isometrically" by means of Grass Instruments FT03 C force-displacement transducers, as previously described for isolated vascular segments (Högestätt et

al., 1983). The resting tension of the preparations was adjusted to between 4 and 6 mN. Following a 60-min accommodation period in standard Krebs solution, the preparations were exposed to a 124 mM K^+ solution (prepared by exchanging all NaCl in the standard Krebs solution with KCl). Preparations responding to K^+ with contractions of an amplitude lower than 10 mN were discarded (approximately 10 % of the ring segments). Responses to NA were always recorded in the continuous presence of cocaine (10 μ M) and propranolol (0.3 μ M).

Release of 3H -NA.

The rabbit urethra was cut open longitudinally, and the urethral mucosa removed by sharp dissection. A longitudinally cut preparation of the urethral smooth muscle, weighing approximately 100 mg, was dissected into eight interconnecting strips. The tissue was subsequently incubated with 3H -NA (1 μ M) in standard Krebs solution (37 °C) for a period of 45 min. In the release studies the standard Krebs solution was supplied with 0.3 mM ascorbic acid and bubbled with 95 % O_2 and 5 % CO_2 . After extensive rinsing in standard Krebs solution (3 x 10 min), the urethral specimen was divided into two symmetrical halves, each consisting of four interconnecting strips. The preparations were placed in separate water-jacketed perfusion chambers (37 °C), each with a volume of 0.4 ml, and further rinsed by superfusion for an additional 60-min period with pre-warmed (37 °C) standard Krebs solution. The perfusion solution, delivered by a peristaltic pump model P3

(Pharmacia), entered the chamber at the bottom and left at the top. The perfusion rate was set to 3.8 ml/min. Two ringformed electrodes, covered by nets of stainless steel, were situated in the upper and lower part of each chamber. The preparations were stimulated electrically with square pulses (0.8 ms duration), delivered at a frequency of 10 Hz by a Grass S88 stimulator. The polarity was altered following each single pulse by a polarity switching unit. The current strength through each chamber was 150 mA, as measured by a Tektronix oscilloscope. The perfusate was collected in scintillation vials by means of Redirac fractional collectors (LKB). The collection time for each sample was 90 s. During the experiment, the tissues were stimulated four times (S_I - S_{IV}) with 15 min intervals, starting with the first stimulation 96 min after the end of the incubation period with ^3H -NA. Each stimulation period lasted for 60 s. The stimulation-induced efflux of ^3H , obtained during three consecutive sampling periods (3 x 90 s) following the beginning of each stimulation, was expressed as a fraction of the total amount of radioactivity remaining in the tissue at the onset of the stimulation period. Increasing concentrations of nifedipine, verapamil, and diltiazem were introduced 13 min before the third (S_{III}) and fourth (S_{IV}) stimulation period, and the fractional release of ^3H in these stimulations were related to that in the second (S_{II}), which was used as a reference. Thus only two concentrations of inhibitor, and in some experiments only one, were tested in each experiment. The ratios between S_{II} and S_{III} or S_{IV} were subsequently

related to the ratios obtained in a series of control experiments ($n=12$), where S_{III} comprised $97 \pm 3 \%$ and S_{IV} $97 \pm 3 \%$ of that in S_{II} . The ratios in the control experiments were set to 100 %. At the end of all experiments, the tissues were removed from the chambers, dissolved in Soluene 350 (Packard), and the 3H activity measured as described below.

Radioligand binding studies

A radioligand binding technique for alpha-adrenoceptors, using 3H -dihydro-alpha-ergocryptine (3H -DHE) as a marker for alpha-adrenoceptors, was used on a crude membrane preparation of the rabbit bladder base and urethra, essentially as previously described (Larsson, 1983). In brief, the rabbit bladder base and urethra were cut open, cleaned from mucosa, and stored frozen at $-80^\circ C$. For binding assays, the pooled muscle preparations were placed in ice-cold radioligand binding buffer (50 mM Tris/HCl, pH 7.5 at room temperature), minced and subsequently homogenized with a Polytron PT10/35 homogenizer (Kinematica). The homogenate was passed through a 0.5 mm nylon mesh and centrifuged at $39\,000 \times g$ for 20 min at $4^\circ C$. The pellet was suspended in fresh buffer and recentrifuged. The resulting final pellet was resuspended in fresh buffer and filtered through a 0.118 mm nylon mesh, and the filtrate used in the binding assay.

Each sample in the assay contained membranes, 3H -DHE, and various concentrations of drugs, or their solvents,

and was incubated in dark environment at 25°C for 60 to 75 min in a final volume of 0.5 ml. At the end of the incubation period 1.5 ml of ice-cold buffer was added to each sample and the membrane suspension rapidly filtered under reduced pressure through a Whatman GF/C glass fiber filter, presoaked with buffer containing 1 mg/ml of bovine serum albumin. The filter was washed with four 5 ml portions of ice cold buffer and then placed in a scintillation vial for measurement of ^3H activity.

From some samples an aliquot of 20 μl was taken and the ^3H content analyzed to determine the radioligand concentration. Specific binding was defined as the difference between total binding and non-specific binding. Non-specific binding was taken as the binding remaining in the presence of 10 μM phentolamine. Protein content was determined by the method of Lowry et al. (1951), using bovine serum albumin (Serva) as standard and radioligand binding buffer as blank.

Treatment of data

Statistical analysis of the results was performed by means of Student's t-test for paired and unpaired data when appropriate. The 0.05 level of probability was accepted as significant. The results shown in tables, figures, and the text are expressed as mean $\pm$ SEM. A subsequent parenthesis following the mean $\pm$ SEM value represents the antilog of the mean value. The method of Cheng and Prusoff (1973) was used to determine the K_i -

values, where $K_i = IC_{50} / (1 + [DHE] / K_{DHE})$. [DHE] is the concentration of 3H -DHE in the assay and K_{DHE} equals the K_D for 3H -DHE computed from saturation binding studies (0.9 nM). In both binding and functional studies the IC_{50} -values were determined graphically for each curve. Linear least square regression analysis was used where appropriate. Outliers were checked for by Dixon's gap test (Bliss, 1967). n = number of determinations. In the binding studies n refers to the number of experiments, each consisting of duplicate or triplicate determinations.

Drugs and chemicals

The following drugs were used: ($\pm$)-noradrenaline hydrochloride, papaverine hydrochloride (Sigma), cocaine hydrochloride (Merck), ($\pm$)-propranolol hydrochloride (ICI), phentolamine methanesulphonate (Ciba-Geigy), nifedipine, nimodipine (Bayer), felodipine (Hässle), ($\pm$)-verapamil hydrochloride (Knoll), and diltiazem hydrochloride (Tanabe). EGTA (ethyleneglycol-bis beta-aminoethyl ether -N,N'-tetraacetic acid) was obtained from Merck, and Tris (Tris(hydroxymethyl) aminomethane) from Sigma.

Nifedipine and nimodipine (1 mg/ml) were provided in ampoules, dissolved in a mixture of ethanol (150 mg/ml) and polyethylene glycol (150 mg/ml) in water. For functional studies, verapamil, and diltiazem were dissolved in water, and noradrenaline, cocaine, propranolol and phentolamine dissolved in 0.9 % NaCl,

containing 1 mM ascorbic acid. Subsequent dilutions of the drugs (including nifedipine) were made with 0.9 % NaCl (supplied with 1 mM ascorbic acid). For binding studies, phentolamine, verapamil, papaverine, and diltiazem were dissolved in water and felodipine dissolved in ethanol; dilutions were made with water for all substances. The drug concentrations reported are the final concentrations, assuming a complete dissociation of the respective salts. Nifedipine, nimodipine, and felodipine were kept dark until used to minimize light-induced degradation.

^3H -(-)noradrenaline (2.7 Ci/mmol) and ^3H -dihydro- α -ergocryptine (21.9 - 30.5 Ci/mmol) were obtained from New England Nuclear. Commercially available scintillation fluids were added to the scintillation vials, and the ^3H activity measured in a liquid scintillation spectrometer (RackBeta 1215, LKB). Counting efficiencies were determined with the external standard channels ratio method, and were found to be approximately 25 and 50 % for release and binding studies, respectively.

Chemicals used were of analytical grade. Redistilled water was used for preparing all solutions.

RESULTS

Studies of mechanical activity

In standard Krebs solution, the female rabbit urethra did not show spontaneous phasic contractile activity. Repeated exposures to a K^+ rich solution (containing 124 mM K^+) every 30 min failed to produce reproducible contractions. In general, the maximum response to K^+ was reduced by between 10 and 20 % following each application of the ion. Phentolamine (1 μ M), introduced into the muscle bath 15 min before the third exposure to K^+ , reduced the K^+ contraction to 27 ± 4 % ($n=12$) of the expected response (Fig 1), which was determined in control experiments run in parallel. In the presence of phentolamine, solutions with K^+ concentrations ranging between 20 and 60 mM (prepared by exchanging various amounts of NaCl in the standard Krebs solution with KCl) induced contractions smaller than those evoked by 124 mM K^+ .

NA induced concentration-dependent contractions in urethral ring preparations (Fig 2). A maximum response, amounting to 122 ± 7 % of a preceding contraction evoked by 124 mM K^+ (first exposure), was obtained at a NA concentration of 0.1 mM. The EC_{50} value was approximately 1 μ M ($\log EC_{50} = -5.94 \pm 0.10$, $n=10$). Preincubation of the preparations in Ca^{2+} -free medium, supplied with 1 mM EGTA, for a period of 10 min abolished the contractile response to 124 mM K^+ ($n=4$), 1 μ M NA ($n=6$), and 10 μ M NA ($n=4$) (Fig 3).

The inhibitory effects of the Ca^{2+} -entry blockers were examined on the contractile response induced by $1\ \mu\text{M}$ NA. Following a control response to NA, the preparations were exposed to increasing concentrations of either nifedipine, verapamil, or diltiazem, and the response to NA determined after each concentration of the drugs. Control experiments, run in parallel, showed that $1\ \mu\text{M}$ NA produced reproducible contractions when repeated 6 times at 30 min intervals. All three Ca^{2+} -entry blockers suppressed the response to NA in a concentration-related fashion (Fig. 4). The following order of potency was found: nifedipine > verapamil $\approx$ diltiazem (Table 1). The maximum inhibitions also differed between the drugs. In contrast to nifedipine and diltiazem, which produced a maximum inhibition of approximately 50 to 60 %, verapamil was able to abolish the NA-induced contraction (Table 1). The inhibitors did not influence the tension of resting urethral preparations.

In a separate series of experiments, the effect of verapamil ($1\ \mu\text{M}$) on the concentration-response curve for NA (added cumulatively) was examined. Although tachyphylaxis is normally encountered following consecutive, cumulative applications of NA in this preparation, verapamil appeared not to competitively antagonize the response to NA, since in preparations exposed to verapamil the maximum of the concentration-response curve for NA was reduced considerably more than in the controls ($n=5$) (not shown).

Release of ^3H -NA.

The effects of nifedipine, verapamil, and diltiazem on the electrically evoked release of ^3H -NA from the rabbit urethra is shown in Fig. 5. As can be seen, nifedipine (0.003 - 3 μM) failed to significantly alter the stimulation-induced efflux of ^3H , as compared to the corresponding controls. However, the release of ^3H was significantly reduced by 19 and 24 % in the presence of 1 and 10 μM diltiazem, respectively. Verapamil (0.1 and 1 μM), on the other hand, significantly increased the efflux of ^3H by 8 and 29 %, whereas 10 μM verapamil failed to significantly alter the ^3H efflux. None of the Ca^{2+} -entry blockers significantly affected the basal efflux of ^3H .

Radioligand binding studies.

Various substances with reported calcium antagonistic properties were tested for their abilities to inhibit alpha-adrenoceptor binding, using ^3H -DHE as a marker for alpha-adrenoceptors (Fig.6). It was found that verapamil and papaverine inhibited alpha-adrenoceptor binding in a concentration-dependent manner. The $\log K_i$ -values were -7.00 ± 0.04 (0.1 μM) for verapamil ($n=6$) and -4.93 ± 0.15 (12 μM) for papaverine ($n=3$). No additional inhibition of ^3H -DHE binding was obtained when verapamil (10 μM) and, phentolamine (10 μM) were added simultaneously, as compared to separate applications of the drugs. Nifedipine ($n=6$), and the structurally related compounds nimodipine ($n=3$) (not shown) and felodipine ($n=3$) (not shown), had virtually no effect on

^3H -DHE binding. Diltiazem ($n=6$) also failed to significantly alter ^3H -DHE binding.

To investigate if the inhibition of ^3H -DHE binding by verapamil was competitive, saturation studies with ^3H -DHE were performed in the absence and presence of verapamil ($0.3\ \mu\text{M}$). The results from these experiments are presented as Scatchard and Hill plots in Fig. 7a and b. Whereas the number of receptors per mg protein (B_{max}) did not significantly differ between the two groups, 101 ± 15 ($n=4$) versus 105 ± 13 fmol/mg protein ($n=5$), the dissociation constant (K_D) for ^3H -DHE was increased in the presence of verapamil from 0.9 ± 0.1 ($n=4$) to 2.8 ± 0.4 nM ($n=5$), as revealed by Scatchard plots. The slopes of the Hill plots were close to unity; the slope factors (n_H) were 0.92 ± 0.05 ($n=4$) and 0.96 ± 0.02 ($n=5$) in the absence and presence of verapamil ($0.3\ \mu\text{M}$), respectively.

In order to elucidate if the effect of verapamil was Ca^{2+} sensitive, the inhibition of ^3H -DHE binding by $0.3\ \mu\text{M}$ verapamil was measured in the absence and presence of EGTA ($0.9\ \text{mM}$) or Ca^{2+} ($1.3\ \text{mM}$). EGTA did not influence specific binding of $1\ \text{nM}$ ^3H -DHE, whereas Ca^{2+} decreased the ^3H -DHE binding to $84 \pm 2\%$ ($n=4$) ($p < 0.01$). No significant difference was seen in the ability of verapamil to inhibit ^3H -DHE binding, in the absence or presence of EGTA or Ca^{2+} , when expressed in per cent of respective control.

DISCUSSION

As shown in the present study, the contractile responses to K^+ (124 mM) and NA (1 and 10 μ M) in the female rabbit urethra were abolished in Ca^{2+} -free medium, indicating that the contractions induced by both K^+ and NA are almost entirely dependent on influx of extracellular Ca^{2+} . Furthermore, phentolamine (1 μ M) suppressed the response to 124 mM K^+ by approximately 73 %. This suggests that the main part of the K^+ contraction is caused by neuronally released NA and subsequent stimulation of postjunctional alpha-adrenoceptors. Thus, it seems that NA may induce contraction by mechanisms independent of the state of polarization of the cell membrane. The small contraction produced by K^+ in the presence of alpha-adrenoceptor blocker together with the lack of spontaneous contractile activity suggest that membrane depolarization is not a major route for muscle activation in the rabbit urethra. These results favour the view that NA stimulates contraction in the rabbit urethra by promoting the entry of Ca^{2+} through receptor-operated Ca^{2+} channels (Bolton, 1979) in the cell membrane and not by releasing intracellularly stored Ca^{2+} or by causing membrane depolarization and subsequent activation of potential-operated Ca^{2+} channels (Bolton, 1979).

Electrophysiological studies on the heart cell have demonstrated that Ca^{2+} -entry blockers ("slow channel blockers" or "organic Ca^{2+} antagonists") more or less

selectively reduce the inward movement of Ca^{2+} ("slow inward current") through voltage-dependent, ion-selective channels ("slow channels") in the muscle membrane (Fleckenstein, 1977; Nayler and Poole-Wilson, 1981). However, different Ca^{2+} -entry blockers appear to affect the slow channels in different ways. For example, the nifedipine-induced inhibition of the slow channel is not accompanied by a change in the kinetics of the channel (Bayer and Ehara, 1978; Kohlhardt and Fleckenstein, 1977), in contrast to the effects of verapamil and D 600 (Nawrath et al., 1977; Bayer and Ehara, 1978; McDonald et al., 1980), but is rather due to a reduction of the number of channels available for Ca^{2+} transport (Bayer and Ehara, 1978). In concentrations higher than those needed for slow channel blockade, D 600 was shown also to interfere with fast Na^+ channels (Galper and Catterall, 1979). These effects of D 600 were stereoselective, the slow channel blocking effect being associated with the (-) enantiomer, whereas the fast channel blocking effect was attributed primarily to the (+) enantiomer of D 600 (Bayer et al., 1975).

It is well known that the movement of Ca^{2+} across the cell membrane is an essential regulator of heart and smooth muscle contraction and of transmitter release in nerve terminals. Depolarization-induced Ca^{2+} influx through tetrodotoxin-resistant, voltage-dependent membrane channels has been demonstrated in a number of excitable tissues, including myocardium, smooth muscle, and nerve tissue (Bolton, 1979; Reuter, 1979; Hagiwara

and Byerly, 1981). There is indirect evidence suggesting that voltage-dependent and receptor-operated Ca^{2+} channels in different tissues vary in their sensitivity to Ca^{2+} -entry blockers. Thus, voltage-dependent Ca^{2+} channels in myocardium and smooth muscle are particularly sensitive to the Ca^{2+} -entry blockers. Voltage-dependent Ca^{2+} channels in vertebrate neurons and receptor-operated Ca^{2+} channels in some smooth muscle preparations, on the other hand, have been found to be relatively resistant to most of these drugs (Bolton, 1979; Hagiwara and Byerly, 1981; Triggle, 1981).

In the present study, the Ca^{2+} -entry blockers nifedipine, verapamil, and diltiazem all inhibited NA-induced contractions in the rabbit urethra. Nifedipine was approximately 25 times more potent than verapamil and diltiazem. In contrast to verapamil, nifedipine and diltiazem failed to abolish the NA-induced contraction, producing maximum inhibitions of 55 and 60 %, respectively. In two previous studies on isolated human mesenteric arteries and veins (Mikkelsen et al., 1979) and perfused rat mesentery (Kondo et al., 1980), verapamil was also noted to produce a larger inhibition of NA-induced contractions than nifedipine. A similar discrepancy between the effects of verapamil and nifedipine was not found for K^{+} -induced contractions (Mikkelsen et al., 1979; Kondo et al., 1980). Possibly, the failure of diltiazem and nifedipine to abolish the contractile response to NA in the rabbit urethra might

reflect the existence of different receptor-operated Ca^{2+} channels in the muscle membrane, one of which is insensitive to nifedipine and diltiazem.

The stimulation-induced efflux of ^3H from adrenergic nerve endings in the rabbit urethra was significantly reduced by diltiazem. The concentrations required to produce this effect were approximately the same as those inhibiting NA-induced contractions. In contrast to diltiazem, nifedipine had virtually no effect on adrenergic transmitter release. A similar lack of effect of the 1,4-dihydropyridine Ca^{2+} -entry blockers nifedipine or nicardipine on transmitter release, in concentrations which inhibited the contractile activity, has previously been demonstrated for adrenergic nerves in vascular smooth muscle and heart, and for cholinergic nerves in intestinal smooth muscle (Starke and Schümann, 1973; Högestätt et al., 1982; Kajiwarra and Casteels, 1983; Kaplita and Triggle, 1983). Thus, in comparison with diltiazem, nifedipine appeared to be a more selective drug with respect to its actions on smooth muscle and peripheral nerves in the rabbit urethra. Notably, low concentrations of verapamil (0.1 and 1 μM) significantly increased the stimulation-induced efflux of ^3H from the rabbit urethra.

In radioligand binding studies, a number of authors have found different effects of nifedipine, verapamil, and diltiazem when used to inhibit ^3H -1,4-dihydropyridine binding to smooth muscle, cardiac, and brain membranes. For example, verapamil was shown only partly or bi-

phasically to inhibit binding of both ^3H -nimodipine and ^3H -nitrendipine, whereas nifedipine competitively antagonized ^3H -1,4-dihydropyridine binding (DePover et al., 1982; Ehlert et al., 1982; Ferry and Glossmann, 1982; Marangos et al., 1982). Diltiazem, on the other hand, was found to stimulate ^3H -1,4-dihydropyridine binding, (DePover et al., 1982; Ferry and Glossmann, 1982). Thus, if, as has been suggested, Ca^{2+} -entry blockers can be used as markers of voltage-dependent Ca^{2+} channels, these results suggest the existence of different subtypes of the Ca^{2+} channel, or the existence of multiple binding sites for Ca^{2+} -entry blockers on each Ca^{2+} channel (Murphy and Snyder, 1982; DePover et al., 1982). Alternatively, the Ca^{2+} -entry blockers may bind to molecular entities different from (DePover et al., 1982) or even unrelated to the Ca^{2+} channels. This might explain the apparent lack of correlation between some binding and functional studies. For example, nifedipine potently inhibits binding of ^3H -nitrendipine or ^3H -nimodipine to rat or guinea-pig brain membranes (Bellemann et al., 1982; Ehlert et al., 1982; Murphy and Snyder, 1982; Marangos et al., 1982), even though it is unable to affect K^+ -induced $^{45}\text{Ca}^{2+}$ uptake into rat brain synaptosomes (Nachshen and Blaustein, 1979) and catecholamine release from vesicular preparations from guinea-pig brain (Ebstein and Daly, 1982).

Although Ca^{2+} -entry blockers are generally considered to interact with membrane channels for Ca^{2+} , and thus influence the excitation-contraction coupling process at

a step subsequent to receptor stimulation, several recent studies suggest additional sites of action for some Ca^{2+} -entry blockers (Church & Zsote^r, 1980; Triggle, 1981). For example, in intact human platelets, verapamil was described both to inhibit adrenaline-induced aggregation and specific binding of ^3H -DHE in a concentration-dependent manner (Barnathan et al., 1982). The inhibition of binding was competitive and occurred in roughly the same concentration range as that inhibiting aggregation. The authors therefore suggested that verapamil, in addition to reducing Ca^{2+} -entry, also may inhibit adrenaline-induced platelet aggregation at the level of the platelet alpha-adrenoceptor. Alpha-adrenoceptor interaction have also been reported for verapamil and the related compound D 600 in a number of other tissues, using different alpha-adrenoceptor radio-ligands (Fairhurst et al., 1980; Glossmann and Hornung, 1980; Jim et al., 1981; van Meel et al., 1981; Nayler et al., 1982, Motulsky et al., 1983).

Amongst the Ca^{2+} -entry blockers examined in the present study (nifedipine, nimodipine, felodipine, verapamil, and diltiazem), only verapamil inhibited the binding of ^3H -DHE to urethral alpha-adrenoceptors. Papaverine also inhibited ^3H -DHE binding with a potency approximately 100 times less than that of verapamil. This is interesting since papaverine is frequently used as a reference dilator in pharmacological studies, and the concentrations used to produce relaxation largely agrees with those found to inhibit ^3H -DHE binding in the rabbit urethra. The inhibitory effect of verapamil was

caused by inhibition of specific binding of ^3H -DHE, since no additional inhibition was obtained when verapamil (10 μM) and phentolamine (10 μM) were added simultaneously, as compared to separate applications of the drugs. The inhibition produced by verapamil appeared to be of the competitive type, since 0.3 μM of verapamil shifted the dissociation constant for ^3H -DHE towards higher concentrations without significantly altering the number of alpha-adrenoceptors, as revealed by Scatchard plots. In addition, the slopes of Hill plots for ^3H -DHE binding in the absence or presence of verapamil were close to unity, indicating a parallel shift of the concentration-response curve for ^3H -DHE. These findings suggest that ^3H -DHE and verapamil compete for the same binding sites.

Assuming competitive antagonism between verapamil and ^3H -DHE for the alpha-adrenoceptors, the K_D -value for verapamil, calculated from the saturation studies with ^3H -DHE, where $K_D = [\text{antagonist}] / \text{concentration ratio} - 1$ (Williams and Lefkowitz, 1977), was found to be 0.1 μM . This is equal to the K_i -value obtained from the inhibition studies. However, the K_i -values for verapamil reported by others (van Meel et al., 1981; Barnathan et al., 1982; Karliner et al., 1982; Nayler et al., 1982; Motulsky et al., 1983) were approximately 6 to 100 times higher than the K_i -value obtained in the present study. This difference does not seem to reflect a selectivity of verapamil for either α_1 - or α_2 -adrenoceptors, since the rabbit urethra contains

predominantly α_2 -adrenoceptors, (Andersson et al., 1983) and the highest K_i -value previously reported (22 μM) was obtained from studies of inhibition of binding to rat brain α_2 -adrenoceptors, using the radioligand ^3H -clonidine (van Meel et al., 1981). Moreover, Motulsky et al. (1983) found similar K_i -values for verapamil to the α_1 - and α_2 -adrenoceptor, when the drug was used to inhibit specific binding of ^3H -prazosin and ^3H -yohimbine, respectively.

In the present study, the interaction between verapamil and the α -adrenoceptors did not appear to be Ca^{2+} -sensitive, since addition of 1.3 mM Ca^{2+} or 0.9 mM EGTA to the incubation medium failed to alter the inhibition of ^3H -DHE binding produced by verapamil, as compared to controls. This is in agreement with the results reported by Fairhurst et al. (1980) and Barnathan et al. (1982), showing that the inhibition of ^3H -DHE and ^3H -WB 4101 binding to human platelet and rat brain membranes by verapamil and the related compound D 600, respectively, were not influenced by increasing the Ca^{2+} concentration of the incubation medium to 10 mM.

The inhibitory effects of verapamil on NA-evoked contractions and enhancement of transmitter release observed in the present study occurred in roughly the same concentration interval as that inhibiting binding of ^3H -DHE to urethral α -adrenoceptors. This makes it tempting to suggest that blockade of pre- and post-junctional α -adrenoceptors might have contributed to the effects of verapamil. Although it is difficult to

distinguish between agonists and antagonists in binding studies, the results from our functional experiments are compatible with an antagonistic action of verapamil on urethral alpha-adrenoceptors. A similar conclusion was reached by Galzin and Langer (1983) when studying the effect of verapamil on electrically evoked ^3H -NA release from rabbit hypothalamic slices. The ability of verapamil to interact with urethral alpha-adrenoceptors may explain why this drug, in contrast to nifedipine and diltiazem, was able to abolish the NA-induced contraction in the rabbit urethra.

It may be concluded that although nifedipine, verapamil and diltiazem are commonly grouped under the same heading as " Ca^{2+} antagonists", "slow channel blockers" or " Ca^{2+} -entry blockers", they display large differences in their profiles of action, and some of the drugs appear to have additional sites of action apart from inhibiting Ca^{2+} influx at the level of the Ca^{2+} channels. This must be taken into consideration when they are used as investigational tools.

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Table 1.

Inhibition of noradrenaline ($1\mu\text{M}$)-induced contractions by nifedipine, verapamil, and diltiazem in the rabbit urethra. Conditions as in Fig. 4.

Drug	n	$I_{\max}$ (%) ^a	Log IC_{50} ^b
Nifedipine	7	55 ± 5	-7.70 ± 0.11 (2.0×10^{-8} M) ^c
Verapamil	9	96 ± 2	-6.29 ± 0.11 (5.1×10^{-7} M) ^c
Diltiazem	6	60 ± 3	-6.31 ± 0.12 (4.0×10^{-7} M) ^c

^aMaximum inhibition obtained $\pm$ SEM

^bLogarithm of the drug concentration (M) producing half maximum inhibition

^cAntilog of the mean



Fig 1. Reduction of the contractile response to K^+ (124 mM) by $1 \mu M$ phentolamine (PhA) in a ring preparation of the rabbit urethra. PhA was introduced to the muscle bath 15 min before the third exposure to K^+ .

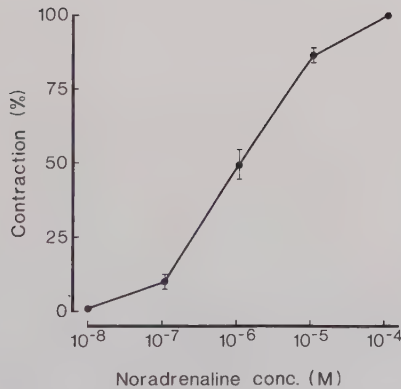


Fig 2. Concentration-response curve for noradrenaline (added cumulatively) in the rabbit urethra. 100 % denotes the maximum response elicited by noradrenaline (25 ± 3 mN). The experiments were performed in the continuous presence of $10 \mu M$ cocaine and $0.3 \mu M$ propranolol. $n=10$.

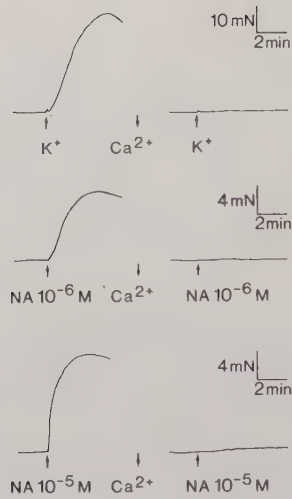


Fig 3. Effect of Ca^{2+} removal on the contractile response to K^+ (124 mM) and noradrenaline (NA) in the rabbit urethra. At downward arrows, the preparations were exposed to a Ca^{2+} -free standard Krebs solution, containing 1 mM EGTA. After 10 min in Ca^{2+} -free medium, the preparations were again exposed to the stimulants. Responses to NA were always obtained in the continuous presence of 10 μ M cocaine and 0.3 μ M propranolol.

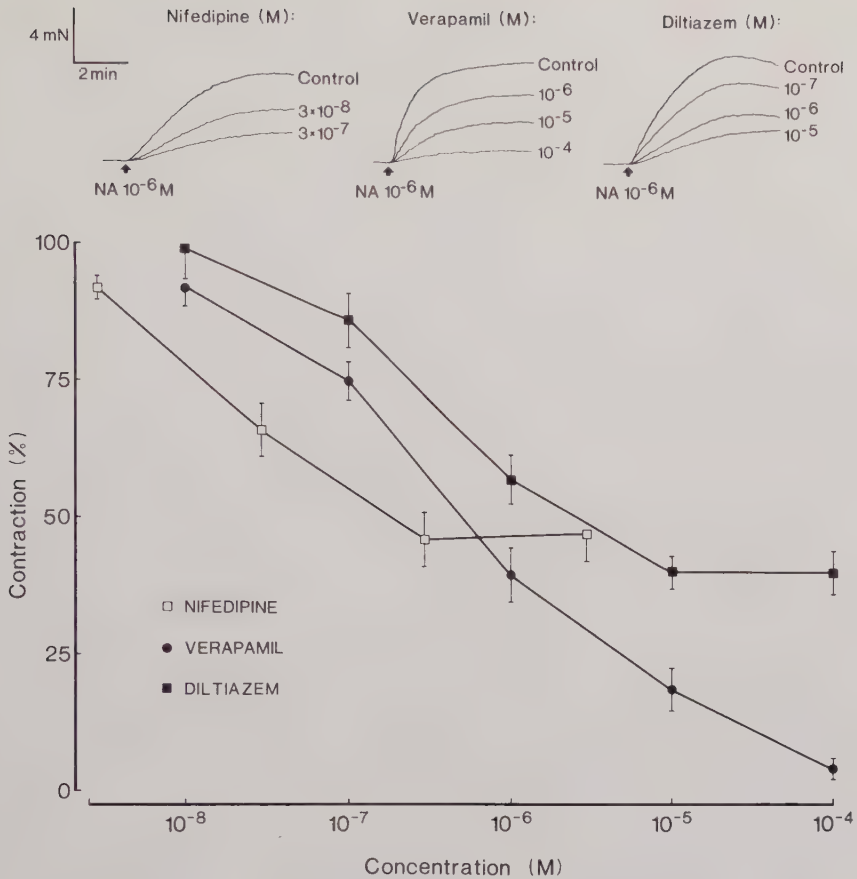


Fig 4. Inhibition of noradrenaline (NA) -induced contractions by nifedipine, verapamil, and diltiazem in the rabbit urethra. The preparations were repeatedly exposed to $1 \mu\text{M}$ NA with 30 min intervals. Each concentration of Ca^{2+} -entry blocker was added 15 min before the subsequent application of NA. Upper section: superimposed tension tracings from three different experiments. Lower section: concentration-response curves for the different Ca^{2+} -entry blockers. The amplitude of the responses to NA obtained immediately before application of the various inhibitors was taken as 100 %. The absolute values of these contractions were $10 \pm 2 \text{ mN}$ ($n=7$), $10 \pm 3 \text{ mN}$ ($n=9$), and $12 \pm 2 \text{ mN}$ ($n=6$) for the experiments with nifedipine, verapamil, and diltiazem, respectively. Cocaine ($10 \mu\text{M}$) and propranolol ($0.3 \mu\text{M}$) were present in all experiments.

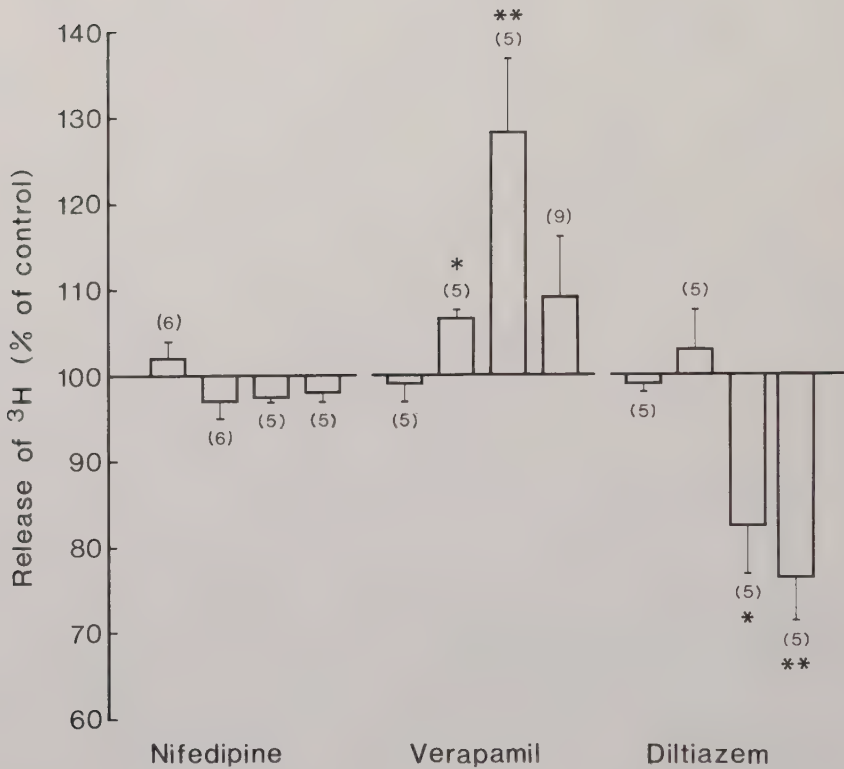


Fig 5. Effects of nifedipine (0.003, 0.03, 0.3, 3 μM), verapamil (0.01, 0.1, 1, 10 μM) and diltiazem (0.01, 0.1, 1, 10 μM) on the electrically evoked efflux of ^3H from the female rabbit urethra, preincubated with ^3H -NA. Figures within brackets indicate the number of experiments. Controls were set to 100 %. *, $p < 0.05$. **, $p < 0.01$. For further explanations, see Methods.

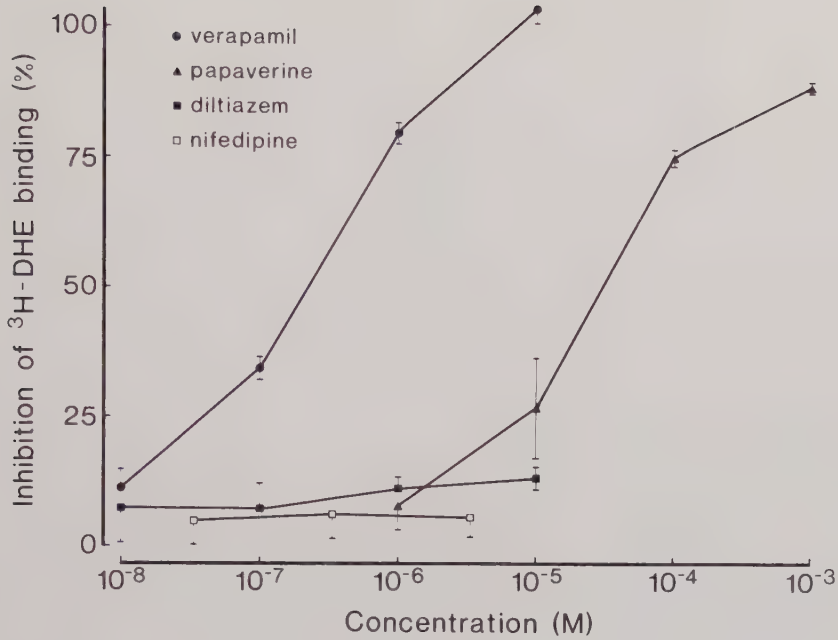


Fig 6. Inhibition of ^3H -DHE binding by verapamil, papaverine, diltiazem, and nifedipine. Membrane suspensions of the rabbit bladder base and urethra were incubated in duplicate with approximately 1 nM ^3H -DHE in the presence of the indicated concentrations of the drugs. The amounts of ^3H -DHE binding inhibited by the various concentrations of the drugs were calculated as a percentage of the amount inhibited by 10 μM phentolamine. $n = 6$ for all substances except for papaverine where $n = 3$.

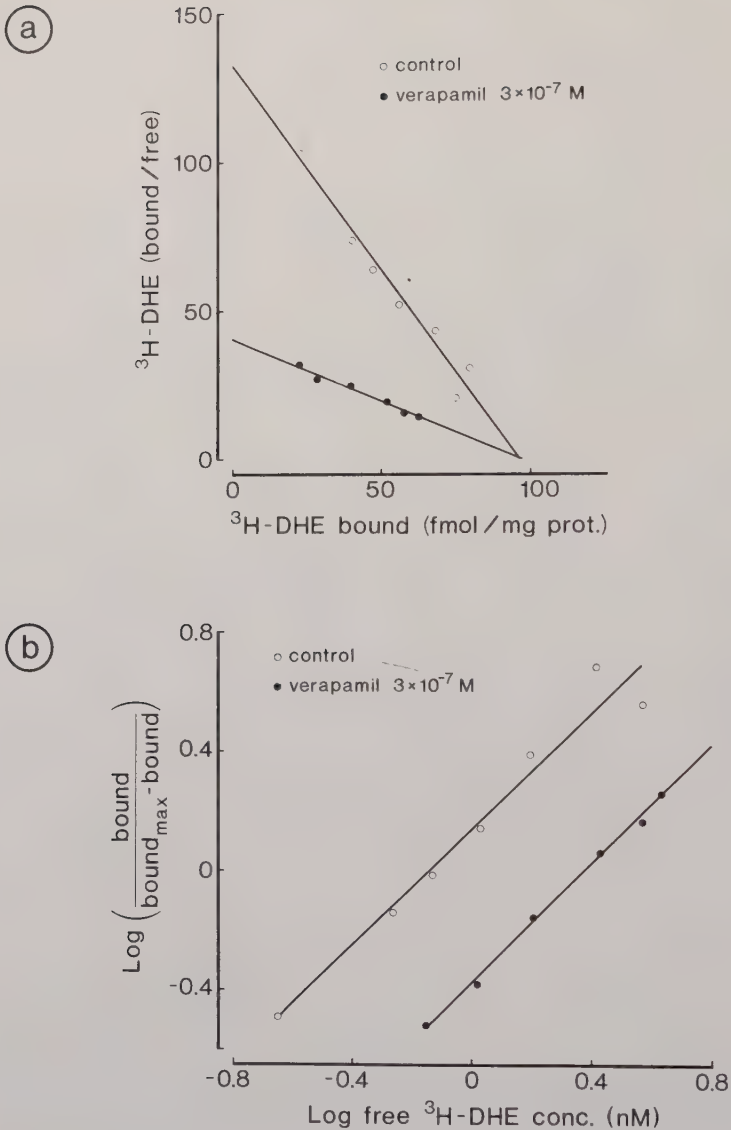


Fig 7. Scatchard (a) and Hill (b) plots for ^3H -DHE binding to membrane preparations of the rabbit bladder base and urethra in the absence (○) and presence (●) of $0.3 \mu\text{M}$ verapamil. For the experiments performed in the absence of verapamil, the range of the ^3H -DHE concentrations was 0.2 to 3.7 nM ($K_D = 0.9 \pm 0.1$ nM), and for those performed in the presence of verapamil, the range of ^3H -DHE concentrations was 0.7 to 4.3 nM ($K_D = 2.8 \pm 0.4$ nM). Each curve is the mean of four experiments in the absence and five experiments in the presence of verapamil.

V

**Influence of antimuscarinics on alpha-
adrenoceptors in the female rabbit urethra**

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Running title: Antimuscarinics and alpha-adrenoceptors

Key words: alpha-Adrenoceptors, antimuscarinics, smooth
muscle contraction, radioligand binding, rabbit urethra

ABSTRACT

The effects of the tertiary amine atropine and its structural analogues homatropine and scopolamine, as well as the quaternary amine emeprone, were evaluated on noradrenaline (NA)-induced contractions of isolated female rabbit urethral ring preparations. In addition, the abilities of these antimuscarinics to inhibit ^3H -dihydro- α -ergocryptine (^3H -DHE) binding to α -adrenoceptors were studied on a crude membrane preparation from the female rabbit bladder base and urethra. Atropine and homatropine depressed the NA-induced contractions in a concentration-dependent way; whereas this was not seen with scopolamine. Emeprone 10^{-5} and 10^{-4} M augmented the contractions, an effect possibly attributable to a NA-uptake blocking effect. All antimuscarinics displaced specific ^3H -DHE binding, the order of potency being atropine > homatropine > emeprone > scopolamine. In general a good correlation was seen between the binding and mechanical activity studies for atropine, homatropine and scopolamine, while this was not found for emeprone.

It is concluded that α -adrenoceptor blockade by atropine can be observed at concentrations exceeding 10^{-7} M. Scopolamine, showing α -adrenoceptor blocking properties only in high concentrations, may be used as an alternative for blockade of muscarinic cholinergic receptors.

INTRODUCTION

The selectivity of a drug is an important feature in pharmacological studies. Even if absolute selectivity hardly is obtainable, many substances are used routinely in various experimental situations as selectively acting "tools". For example, to reduce the influence of cholinergic neurons, atropine is frequently utilized when studying the effects of the sympathetic nervous system on the isolated rabbit urethra. In our laboratory, it was observed that contractions induced by exogenously administered NA were reduced by atropine. We were interested to determine the cause of this depression and if it was a common feature of antimuscarinics. It has previously been suggested that atropine, in addition to its well known antimuscarinic activity, interacts with alpha-adrenoceptor mediated responses in e.g. vascular smooth muscle (cf. Nedergaard & Schrold 1977, Abraham et al. 1981). We therefore assessed the effects of various antimuscarinics on NA-induced contractile responses as well as on alpha-adrenoceptor radioligand binding in the female rabbit urethra.

MATERIALS AND METHODS

Dissection

Female rabbits of the Danish Land race, weighing approximately 3 kg, were sacrificed by injecting air into an ear vein. The abdominal cavity was opened, and the bladder and urethra removed en bloc.

Recording of mechanical activity

After removal of periurethral fat and connective tissue, 2 to 3 ring-formed segments, approximately 3 mm wide, were taken from each urethra. The preparations thus obtained were transferred to organ baths containing Krebs solution (composition, see below) maintained at 37° C and bubbled with a mixture of 95 % O₂ and 5 % CO₂ to give a pH of approximately 7.4. Each preparation was suspended between two parallel L-shaped steel hooks, one of which was connected to a Statham FT03 C force transducer for registration of "isometric" tension. The tension of the preparations could be adjusted by altering the distance between the two hooks. The urethral rings were repeatedly stretched and then allowed to relax until a stable tension level of 4 - 6 mN was obtained. The preparations were left to equilibrate for a period of approximately 1 h before any drugs were added.

In a previous study (Andersson et al. 1983) it was shown that the EC₅₀-value for the contractant effect of NA in the rabbit urethra was approximately 10⁻⁵ M. In the present study this concentration of NA was used to induce contractions.

The inhibitory effects of various antimuscarinic agents were assessed on repetitive NA-induced contractions. The time between each contraction was 20 minutes. Two initial NA-induced contractions were used as controls, and the preparations were then exposed to increasing

concentrations of one of the antimuscarinic agents. The contact time for the drugs was 15 min. The response to NA was determined after exposure to each concentration of the antimuscarinics.

Radioligand binding studies

Membrane preparations

The radioligand binding studies were performed essentially as described previously (Larsson 1983). The bladder base and urethra were cut open, and the mucosa, periurethral fat and connective tissue removed. The remaining tissue was stored frozen at -80°C for up to one month. For binding experiments, pooled tissue was thawed and carefully minced with a pair of scissors and subsequently homogenized with a Polytron PT 10/35 (Kinematica) homogenizer in ice cold buffer containing 50 mM Tris-HCl (pH 7.5 at room temperature).

The homogenate was filtered through a 0.5 mm nylon mesh and centrifuged at $39\,000 \times g$ for 20 min at 4°C . The pellet obtained was suspended in fresh buffer and re-centrifuged. The subsequent final pellet was resuspended in fresh buffer and filtered through a 0.118 mm nylon mesh and the filtrate was used in the binding experiments.

Binding assay

Membranes, ^3H -dihydro- α -ergocryptine (^3H -DHE), and solutions of drugs or their solvents were incubated in triplicate for saturation experiments and in duplicate

for inhibition studies in a total volume of 0.5 ml at 25°C for 60 - 75 min in dark environment. The incubation was terminated by diluting the samples with 1.5 ml of ice cold buffer followed by rapid filtration under reduced pressure through Whatman GF/C glass fibre filters, presoaked with buffer containing 1 mg/ml bovine serum albumin. The filters were rapidly washed four times with 5 ml portions of ice cold buffer.

The filters were placed in scintillation vials and the tritium content determined in a liquid scintillation spectrometer (LKB RackBeta 1215). The counting efficiency was about 50 % as determined with the external standard channels ratio method.

Specific binding was defined as the binding exceeding that in blanks containing 10^{-5} M phentolamine. To determine the concentration of radioactive ligand in the assay, an aliquot of 20 μ l was taken from some tubes and counted for tritium activity. An estimation of free radioligand concentration in the saturation experiments was obtained from the difference between added and bound ^3H -ligand. Protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard and 50 mM Tris-HCl buffer as blank.

Treatment of data

Studies of mechanical activity

Because a certain degree of tachyphylaxis of the urethral rings was observed after repetitive

administration of 10^{-5} M NA, a series of control experiments were performed. Using the mean of the two initial contractions as a reference, the subsequent contractions amounted to $94 \pm 2\%$ ($n=11$), $88 \pm 3\%$ ($n=11$), $81 \pm 3\%$ ($n=11$), and $76 \pm 4\%$ ($n=10$) of the reference value. Hence after the initial two NA-induced responses a reduction of each contraction of approximately 6 % was seen. This tachyphylaxis was compensated for when the effects of the antimuscarinics were determined.

Binding studies

The mean values from duplicate or triplicate determinations were used for calculations. Linear least square fit of lines to the various plots were used where appropriate. IC_{50} -values were determined graphically for each curve. K_i -values from inhibition experiments were determined by the method of Cheng & Prusoff (1973), while the K_D -value for atropine from saturation studies were calculated according to Williams et al. (1977). Outliers were checked for by Dixon's gap test (Bliss 1967).

Statistical analysis of the results was performed by means of Student's t-test for paired data. The 0.05 level of probability was accepted as significant. The results shown in tables and figures are expressed as mean $\pm$ SEM. For studies of mechanical activity n denotes the number of urethral rings tested, whereas in binding studies n states the number of experiments performed.

Solutions and drugs

The following drugs were used: ^3H -dihydro- α -ergocryptine (30.5 Ci/mmol) (New England Nuclear), phentolamine methanesulphonate (Ciba-Geigy), ($\pm$)-nor-adrenaline hydrochloride (Sigma), carbamylcholine chloride (carbachol) (BDH chemicals), atropine sulphate and (-)-scopolamine hydrochloride (Sigma), homatropine hydrochloride (Serva), and emepronium bromide (emeprone) (Kabi).

All chemicals used were of analytical grade. Redistilled water was used for preparing all solutions.

The Krebs solution used in the studies of mechanical activity had the following composition (in mM): NaCl 119, KCl 4.6, CaCl_2 1.5, MgCl_2 1.2, NaHCO_3 15, NaH_2PO_4 1.2, glucose 11. All substances were dissolved and serially diluted in 0.9 % NaCl containing 1 mM ascorbic acid.

For binding studies all substances were dissolved and serially diluted in redistilled water.

RESULTS

Studies of mechanical activity

Scopolamine and the structurally similar anti-muscarinics atropine and homatropine differed in their effects on the $\text{NA}(10^{-5} \text{ M})$ -induced contractions of the

female rabbit urethra (Fig. 1). Scopolamine 10^{-7} - 10^{-4} M had no depressant effect, whereas homatropine (10^{-7} - 10^{-4} M) and atropine (10^{-7} - 10^{-5} M) depressed the contractions in a concentration related way. In concentrations of 10^{-7} and 10^{-6} M, the quarternary ammonium compound emeprone did not influence the urethral contractions induced by NA (10^{-5} M), whereas in concentrations of 10^{-5} M and 10^{-4} M, emeprone augmented the contractions, the lower concentration being the more active (Fig. 1). The cholinceptor stimulating agent carbachol 10^{-4} M and 10^{-3} M, did not influence the NA (10^{-5} M)-induced contractions (n=5) (not shown).

Of the antimuscarinics investigated, only atropine in a concentration of 10^{-4} M per se induced contractions of the urethral rings.

Binding studies

Atropine, homatropine, emeprone, and scopolamine, as well as carbachol, were tested for their abilities to displace alpha-adrenoceptor binding, using ^3H -DHE (1nM) as a marker for alpha-adrenoceptors (Fig. 2, Table 1). All antimuscarinics displaced ^3H -DHE concentration-dependently, while carbachol failed to influence ^3H -DHE binding. Among the antimuscarinics, atropine was 70 times more effective in inhibiting ^3H -DHE binding than scopolamine. Homatropine and emeprone were 4 and 12 times less potent than atropine, respectively.

Being the most effective of the antimuscarinics tested

to inhibit alpha-adrenoceptor binding, atropine was chosen for further studies. To investigate whether the inhibition of ^3H -DHE binding by atropine was competitive, saturation studies with ^3H -DHE were performed on the same membrane preparation in the absence and presence of atropine (10^{-5} M) ($n=4$). The results from these studies are presented in Fig. 3 a and b. The K_D for ^3H -DHE in the saturation studies was significantly ($p<0.05$) increased in the presence of atropine, from 0.4 ± 0.03 nM to 3.1 ± 0.8 nM. The B_{max} -values obtained in the absence of atropine (99 ± 10 fmol/mg protein) was not significantly different from those obtained in the presence of atropine (123 ± 22 fmol/mg protein). The slope factors from Hill plots were both close to unity, being 0.96 ± 0.04 in the absence and 0.97 ± 0.005 in the presence of atropine. The K_D -value for atropine, obtained from the saturation studies, was 1.5×10^{-6} M.

No additional inhibition of ^3H -DHE binding was observed when any of the antimuscarinics was added in high concentrations together with 10^{-5} M phentolamine, as compared to separate application of 10^{-5} M phentolamine.

DISCUSSION

The tertiary amines atropine and homatropine depressed NA-induced contractions of the female rabbit urethral preparations in a concentration-dependent way, whereas this was not seen with scopolamine. Thus the small

structural dissimilarity between these drugs resulted in a large difference in the effect on the contractile responses to NA. This suggests that the interaction was specific in nature. To investigate if the effect was at the level of the alpha-adrenoceptor, radioligand binding studies were performed. The binding studies revealed a 70-fold potency difference between atropine and scopolamine in the ability to inhibit ^3H -DHE binding. The potency of homatropine was 4 times less than that of atropine. The inhibitory effect of atropine appeared to be of the competitive type, as judged from the Scatchard and Hill plots. The K_D -value for atropine, obtained from the saturation studies, was 1.5×10^{-6} M. This is nearly identical to the K_i -value of 2.0×10^{-6} M for atropine revealed from the inhibition studies. Thus, in two separate ways similar dissociation constants were obtained for atropine to the alpha-adrenoceptor in membranes prepared from the female rabbit bladder base and urethra, using the non-selective alpha-adrenoceptor antagonist ^3H -DHE as a marker for alpha-adrenoceptors. These values are in accordance with those reported by others (U'Prichard et al. 1977, Abraham et al. 1981), who found K_i -values for atropine in inhibiting the binding of the selective α_1 -adrenoceptor antagonist ^3H -WB 4101 to membranes prepared from brain tissue similar to those observed in the present study. However, atropine was virtually ineffective in binding to α_2 -adrenoceptors labelled by ^3H -clonidine (U'Prichard et al. 1977, Abraham et al. 1981). In a previous study we have found an α_2 -adrenoceptor content of 75-80 % in

our membrane preparation (Andersson et al. 1983). Possibly the differences in binding of atropine to α_2 -adrenoceptors found in the present study and those of U'Prichard et al. (1977) and Abraham et al. (1981) could be attributed to the use of an antagonist (^3H -DHE) and an agonist (^3H -clonidine), respectively, to label the α_2 -adrenoceptor.

The observation that the antimuscarinics in high concentrations together with 10^{-5} M phentolamine inhibited binding to the same extent as 10^{-5} M phentolamine alone, strengthens the suggestion that these agents and ^3H -DHE compete for the same α -adrenoceptor binding site.

Assuming an interaction between atropine and the α -adrenoceptor according to the law of mass action, the receptor occupancy of atropine to this receptor can be described by the equation: fractional receptor occupancy = $L/(K_D + L)$ (Ruffolo 1983), where L equals the atropine concentration, and K_D stands for the dissociation constant for atropine to the α -adrenoceptor found in saturation radioligand binding studies (1.5×10^{-6} M). From this relationship it could be calculated that atropine (10^{-7} M) would occupy about 6 % of the α -adrenoceptor population whereas the corresponding values for atropine 10^{-6} M and 10^{-5} M were 40 % and 87 %, respectively.

Although a strict comparison between the mechanical and binding studies is not possible in these studies, an occupancy of the α -adrenoceptor population of 40 %

in the presence of atropine 10^{-6} M suggests that atropine in this concentration would influence also the NA-induced contractions. Indeed, a depression of approximately 20 % of the NA-induced contractions was observed in the presence of 10^{-6} M atropine. In general, a good correlation was seen between the binding and mechanical activity studies for atropine and its structural analogues homatropine and scopolamine. The potency order in both types of studies was atropine > homatropine >> scopolamine. This order of potency agrees with that found for these substances in inducing hypotension through alpha-adrenoceptor blockade in conscious, unanaesthetized rats (Cantor et al. 1983).

A structurally different antimuscarinic, the quarternary amine emeprone, in concentrations of 10^{-5} M and 10^{-4} M was found to increase the contractions induced by 10^{-5} M NA. However, in the radioligand binding studies, a small inhibitory effect of emeprone 10^{-5} M was seen, while emeprone 10^{-4} M inhibited ^3H -DHE binding by approximately 60 %. A similar displacement capacity of ^3H -DHE binding was seen with atropine 10^{-5} M. This concentration of atropine reduced the NA-induced contraction with approximately 60 %, whereas emeprone 10^{-4} M increased it. This, together with a lack of agonistic properties, suggest that the contraction-increasing effect of emeprone was not exerted by a direct action on the alpha-adrenoceptors of the urethral smooth muscle. As the effect was maximum at a concentration where only a slight displacement of the ^3H -DHE binding could be

demonstrated, it cannot be excluded that it was attributable to an inhibitory effect on NA uptake mechanisms. Such an inhibitory effect, resulting in an increased NA response (deviation supersensitivity, Westfall 1981), would be counteracted by displacement of NA from alpha-adrenoceptor sites at high concentrations of emeprone.

The results of the present study underline the importance of choosing a drug with the desired effect, and using it in appropriate concentrations. Although the affinity of atropine to muscarinic cholinceptors has been reported to be much higher than the affinity to the alpha-adrenoceptors found in this study (Gilbert et al. 1979, Estrada & Krause 1982, Hammer & Giachetti 1982), high concentrations of atropine are sometimes needed to abolish cholinceptor-mediated responses. Therefore it seems important to determine the concentration range within which a drug has a selective activity. Under the described assay conditions, an atropine concentration not higher than 10^{-7} M is suggested in order to avoid alpha-adrenoceptor effects of atropine. Alternatively scopolamine could be used, since this drug showed alpha-adrenoceptor blocking properties only in significantly higher concentrations than did atropine.

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Table 1

Inhibition constants (K_i) for various antimuscarinics on alpha-adrenoceptor binding in the female rabbit urethra.

	Substances	$\log K_i$ (M)	mean K_i (M)
Tertiary amines	Atropine	-5.70 ± 0.11	2.0×10^{-6}
	Homatropine	-5.08 ± 0.07	8.3×10^{-6}
	Scopolamine	-3.86 ± 0.05	1.4×10^{-4}
Quarternary amine	Emeprone	-4.64 ± 0.06	2.3×10^{-5}

Values are given as mean $\pm$ SEM for $\log K_i$; $n = 6$ for all substances.

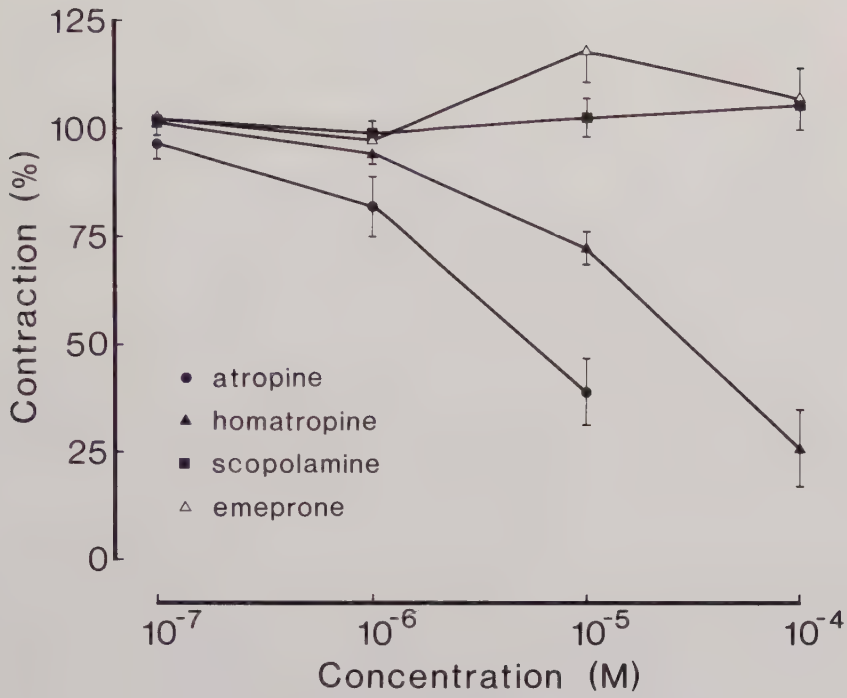


Fig 1. Inhibition of noradrenaline (NA)-induced contractions by the indicated concentrations of atropine, homatropine, scopolamine, and emeprone. For calculations, see methods. $n=5-8$.

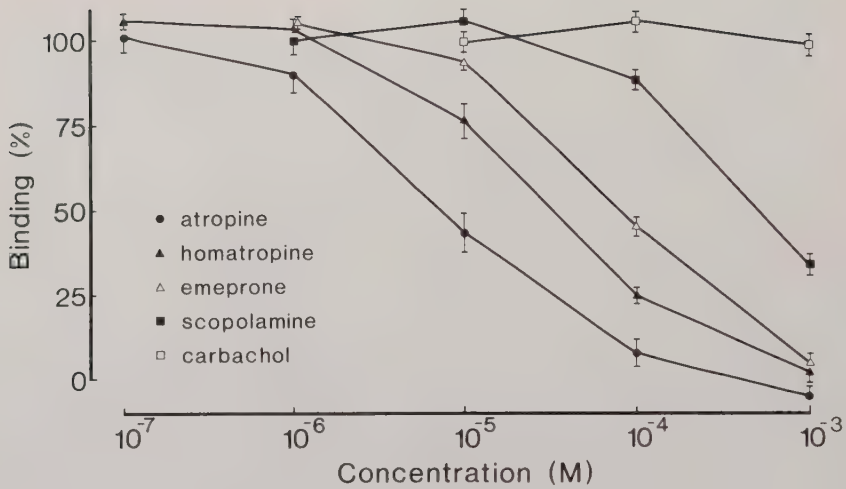


Fig 2. Inhibition of ^3H -DHE binding by the antimuscarinics atropine, homatropine, emeprone, and scopolamine and the cholinceptor agonist carbachol. Membrane suspensions of the rabbit bladder base and urethra were incubated in duplicate with approximately 1 nM ^3H -DHE in the presence of the indicated concentrations of the drugs. The amount of ^3H -DHE bound in the absence of drugs were set to 100 %, and the amount bound in the presence of 10 μM phentolamine were set to 0 %. n=6.

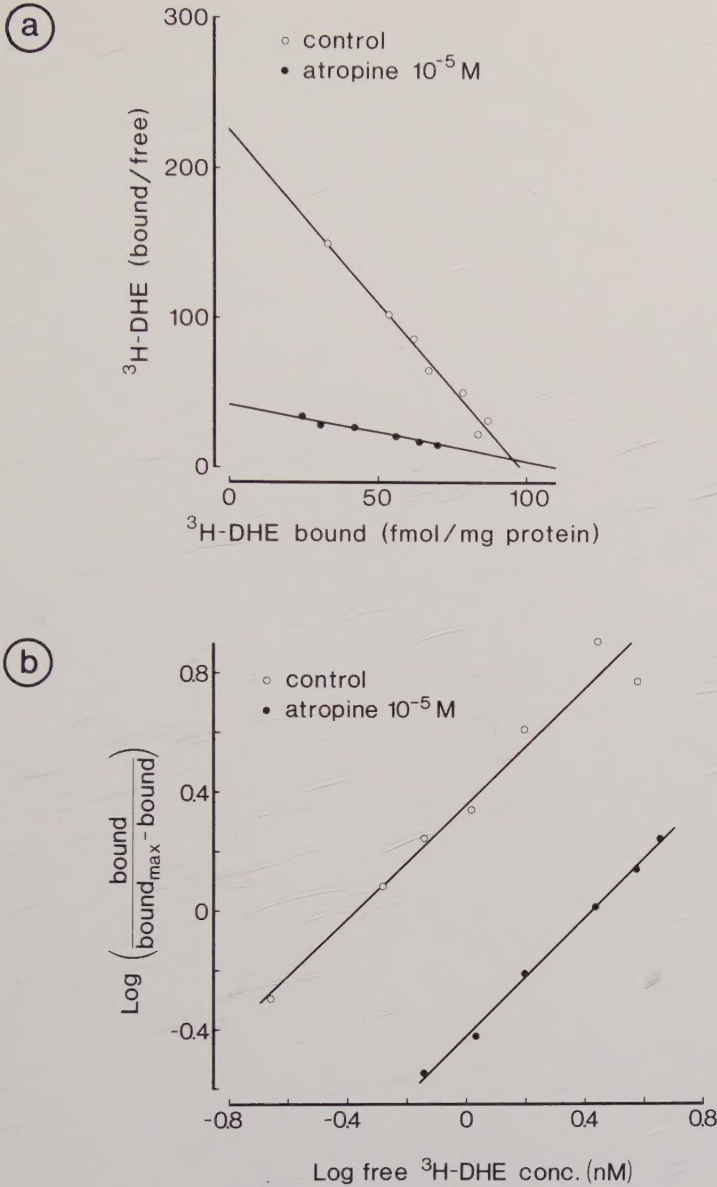


Fig 3. Scatchard (a) and Hill (b) plots for $^3\text{H-DHE}$ binding to membrane preparations from the rabbit bladder, base and urethra in the absence ($\circ$) and presence ($\bullet$) of 10^{-5} M atropine. For the experiments performed in the absence of atropine the range of $^3\text{H-DHE}$ concentrations was $0.2 - 3.8\text{ nM}$, and for those performed in the presence of atropine, the range of $^3\text{H-DHE}$ concentrations was $0.7 - 4.5\text{ nM}$. For each plot the mean of 4 experiments were used.

